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**BARBARA CASSANI**

**MOLECULAR MECHANISMS OF IMMUNE DEFICIENCY IN  
ADENOSINE DEAMINASE (ADA)-DEFICIENT SCID PATIENTS:  
IMPLICATIONS FOR STEM CELL GENE THERAPY**

**PhD Thesis submitted in partial fulfilment of the requirements of the  
Open University for the degree of Doctor of Philosophy  
in Molecular and Cellular Biology**

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**Sponsoring establishment:**

**San Raffaele Telethon Institute for Gene Therapy  
(HSR-TIGET)**

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All the work presented in this thesis has been performed at the San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), in the laboratory directed by Alessandro Aiuti and Maria Grazia Roncarolo.



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**Abbreviations used in this thesis (unless otherwise stated in the text)**

ADA	Adenosine Deaminase
Ado	Adenosine
dAdo	2'-deoxyadenosine
mAb(s)	Monoclonal antibody(ies)
Ag(s)	Antigen(s)
Ig	Immunoglobulin
IVIg	Intravenous Immune Globulin
PBL	Peripheral Blood Lymphocytes
NK	Natural Killer
HSC	Hematopoietic Stem/progenitor Cells
GT	Gene Therapy
LV	Lentiviral Vector
RV	Retroviral Vector
RIS	Retroviral Insertion Site
bp	Base pair
cpm	Counts per millions
FACS	Fluorescence Activated Cell Sorter

## DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were generated by myself, with the exception of the Affymetrix HG-U133A Gene Chip arrays, which were performed in collaboration with Fabrizia Urbinati and Fulvio Mavilio at the Department of Biomedical Sciences, University of Modena and Reggio Emilia.

All sources of information are acknowledged by means of reference.

Some of the results presented and discussed here are contained in the following articles:

A. Aiuti, B. Cassani, G. Andolfi, M. Mirolo, L. Biasco, A. Recchia, F. Urbinati, C. Valacca, S. Scaramuzza, M. Cazzola, D. Sartori, A. Ambrosi, C. Di Serio, M.G. Roncarolo, F. Mavilio, and C. Bordignon. "Multilineage hematopoietic reconstitution without clonal selection in ADA-SCID patients treated with stem cell gene therapy". *J. Clin. Invest. In press*

B. Cassani, M. Mirolo, F. Cattaneo, U. Benninghoff, F. Carlucci, A. Tabucchi, C. Bordignon, M. G. Roncarolo and A. Aiuti. "Deoxyadenosine Inhibits T-cell Activation in ADA-SCID Patients Through a cAMP/PKA-Dependent Pathway Initiated by A<sub>2A</sub> Adenosine Receptor". *Submitted*.

## ABSTRACT

Mutations in the Adenosine Deaminase (ADA) gene are responsible for a form of Severe Combined Immunodeficiency (SCID), explained by the lymphotoxic accumulation of ADA substrates, adenosine and 2'-deoxy-adenosine. Haematopoietic stem cells (HSCs)-based gene therapy (GT) has been shown to correct both the immune and metabolic defect of ADA-SCID children, thus providing a unique model to investigate the molecular mechanisms linking the altered purine metabolism to T cell dysfunction. We found that severely compromised effector functions in ADA-deficient T cells associate with an intrinsically reduced ERK1/2 signaling, a defective activation of CREB and possibly to an altered nuclear recruitment of NF- $\kappa$ B, as predicted by the decreased phosphorylation of I $\kappa$ B $\alpha$ . Conversely, in T-cell lines generated from patients after GT, the biochemical events following TCR-triggering occur properly, leading to restored effector functions. Remarkably, in ADA-deficient but not in gene-corrected T cells, exposure to 2'-deoxy-adenosine induces a strong inhibition of T cell activation. This effect is consistent with an aberrant A<sub>2A</sub> receptor-mediated signaling and PKA hyperactivation.

To assess the long-term safety of GT, retroviral integration sites (RIS) were cloned and analyzed from transduced bone marrow-derived CD34<sup>+</sup> cells before transplantation and from their multilineage progeny in five patients. RIS occur preferentially around transcription start sites and in gene-dense regions, favoring genes transcriptionally active in CD34<sup>+</sup> cells at the time of transduction as well as genes expressed in T cells. However, preliminary quantitative transcript analysis, at clonal cell level, showed no significant alteration in expression of genes correlated with the nearby vector integration, in agreement with the normal gene expression profile and functional behavior of T cell population.

Altogether, these results contribute to extend the present knowledge on the pathogenesis of ADA-SCID and demonstrate the efficacy and safety of HSC gene therapy as a treatment for this disease.

## **CHAPTER 1-Introduction**

### **1.1 Severe Combined Immunodeficiency (SCID)**

Severe Combined Immunodeficiency (SCID) consists of a group of heterogenic genetic disorders characterized by a block in T lymphocyte differentiation that is variably associated with abnormal development of other lymphoid lineages, i.e. B or NK lymphocytes or more rarely of the myeloid lineage (Fischer et al., 1997). The overall incidence is estimated to be on the order of 1:75,000 live births (Buckley et al., 1997). Patterns of inheritance, immunological characteristics, and more recently genotyping have led to the detection of 10 SCID conditions (Table I). Four main pathogenetic mechanisms have been identified:

1. Defective cytokine-dependent survival signaling in T and sometimes Natural Killer (NK) cell precursors. This mechanism causes the most frequent type of SCID (45-60% cases). Deficiency in expression or function of the  $\gamma_c$  cytokine receptor subunit shared by the receptors of interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21 causes X-linked form of SCID (SCID-X1), characterized by absence of both T and NK lymphocytes (Notarangelo et al., 2000). Deficiency in Janus kinase 3 (JAK-3), which is normally associated with the cytoplasmic region of  $\gamma_c$ , results in identical phenotype. Deficiency in the IL-7R $\alpha$  subunit only impairs T cell development (Candotti et al., 2002).
2. Defective V(D)J rearrangement of T cell receptor (TCR) and B cell receptor (immunoglobulin genes). This group accounts for 25-30% of SCID. It results in a T- B- NK+ phenotype. Deficiency in *RAG-1* or *RAG-2*, the lymphoid-

specific recombination initiating elements, or in Artemis, a factor involved in the non-homologous end-joining repair pathway, lead to defective V(D)J rearrangements and thereby death of thymocytes and pre-B cells (de Villartay et al., 2003).

3. Defective purine metabolism. Deficiency of Adenosine Deaminase (ADA) affects all lymphocyte lineages (T- B- NK-). Because ADA is ubiquitously expressed, it is thought that the additional metabolic toxicity can contribute to the poor prognosis of ADA deficiency compared to the other forms of SCID.
4. Defective pre-TCR and TCR signaling. Rare cases of pure T cell deficiency (1%-2%) are caused by defects in either a CD3 subunit, such as CD3 $\delta$  or CD3 $\epsilon$ , or the CD45 tyrosine phosphatase (de Saint Basile et al., 2004; Kung et al., 2000).

For some cases of SCID, the molecular basis remains unknown. These include a few pure T cell deficiencies as well as the so-called reticular dysgenesis condition. It consists of a profound deficiency in T and NK cells and a milder B cell deficiency. Reticular dysgenesis has an autosomal recessive inheritance. Putative key factors in hematopoiesis are probably involved.

Disease		Affected cells	Gene Product
Reticular dysgenesis	AR	T, B, NK, myeloid cells, platelets	Unknown
Alymphocytosis	AR	T, B	<i>RAG-1</i> <i>RAG-2</i>
	AR	T, B	Artemis
Absence of T lymphocytes and NK	X-L	T, NK	$\gamma$ c chain
	AR	T, NK	JAK-3
Absence of T lymphocytes	AR	T	IL-7R $\alpha$ chain
	AR	T	CD45
	AR	T	CD3 $\delta$
ADA deficiency	AR	T, B, NK	ADA

**Table I. Classification of severe combined immunodeficiencies.** AR, autosomal recessive; X-L, X-linked recessive.

### 1.1.1 Adenosine Deaminase (ADA)-deficient SCID: an overview

In 1972, ADA-deficiency was the first of the human immunodeficiency diseases for which the underlying genetic and biochemical defects were identified (Giblett et al., 1972), revealing the crucial importance of this purine catabolic enzyme for the immune development. ADA-deficiency represents the cause of approximately 15-20% of all cases of SCID (Buckley et al., 1997; Kalman et al., 2004), with an overall prevalence ranging from 1:375.000 to 1:660.000 live birth (Buckley et al., 1997). The clinical spectrum of ADA-SCID is quite broad, including severe and milder phenotypes according with the severity of genetic mutations, the residual ADA activity and the extent of the metabolic poisoning, due to the accumulating ADA natural substrates, Adenosine (Ado) and Deoxy-adenosine (dAdo) (Arredondo-Vega et al., 1998). The most relevant clinical manifestations include panlymphocytopenia and impaired



humoral and cellular-mediated immune responses, which are the cause of recurrent opportunistic, often life-threatening, infections. Although the metabolic basis for immune deficiency has largely been established, the causal link between altered purine metabolism and the immunological defect has not been clarified. This is the reason why ADA-SCID has been, and continues to be, the object of several studies aimed to unravel the disease pathogenesis and the role of purine metabolism in the immune system.

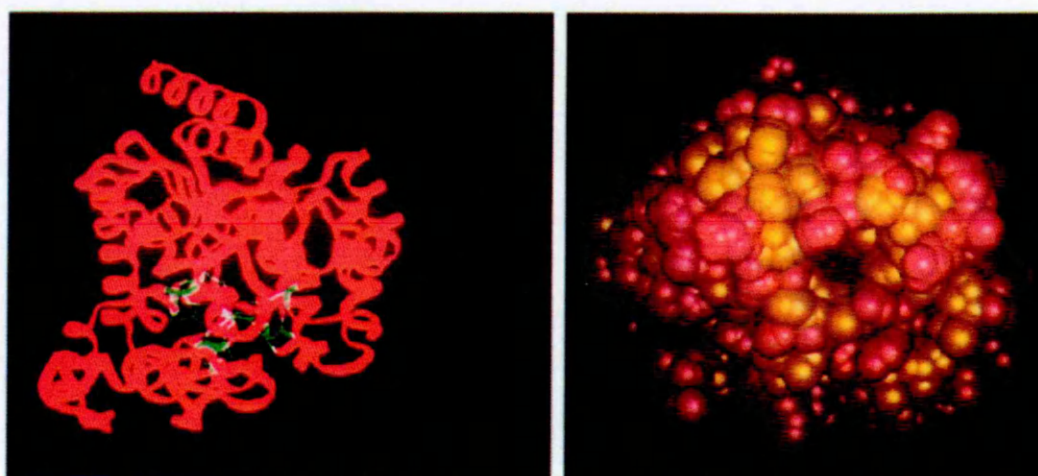
## **1.2 ADA: molecular organization of the gene**

The human gene for adenosine deaminase (ADA, adenosine aminohydrolase) was found to be located in the long arm of chromosome 20 at 20q12-q13.1 (Tischfield et al., 1974) and it was subsequently isolated by positional cloning (Wiginton et al., 1986). The ADA gene is constituted by 12 exons and 11 introns. The promoter region lacks the “TATA” and “CAAT” sequences and is extremely G/C rich, with a binding site for the transcription factor Sp1. Two regulatory sequences driving the tissue specific expression have been identified in the large (15Kb) first intron and at the end of exon 1 (Aronow et al., 1989). The enhancer in the first intron, responsible for the high expression in T cells, displays consensus sequences for binding sites of ADA NF-1 and ADA NF-2 factors, AP1, TCF1 $\alpha$ ,  $\mu$ E and proteins belonging to the Ets transcription factor family. The regulatory region at the end of exon 1 might function as a transcriptional arrest 10 bp long beyond the translational initiation codon in many tissues, such as liver expressing low levels of ADA (Chen et al., 1990). The gene is highly enriched in repetitive sequences of Alu type (23 copies for 18% of total sequence), all but one located in the first 3 introns or in the 5' flanking region, implicated in causing deletions in ADA gene. The mRNA is only 1497 nucleotides long and limited information is available on its post-transcriptional regulation. In cell differentiation studies, it has been demonstrated that ADA mRNA and protein levels

changed while transcription remained constant. The mechanism of this regulation is still not known (Berkvens et al., 1987).

### 1.3 ADA: the enzyme and its role in purine metabolism

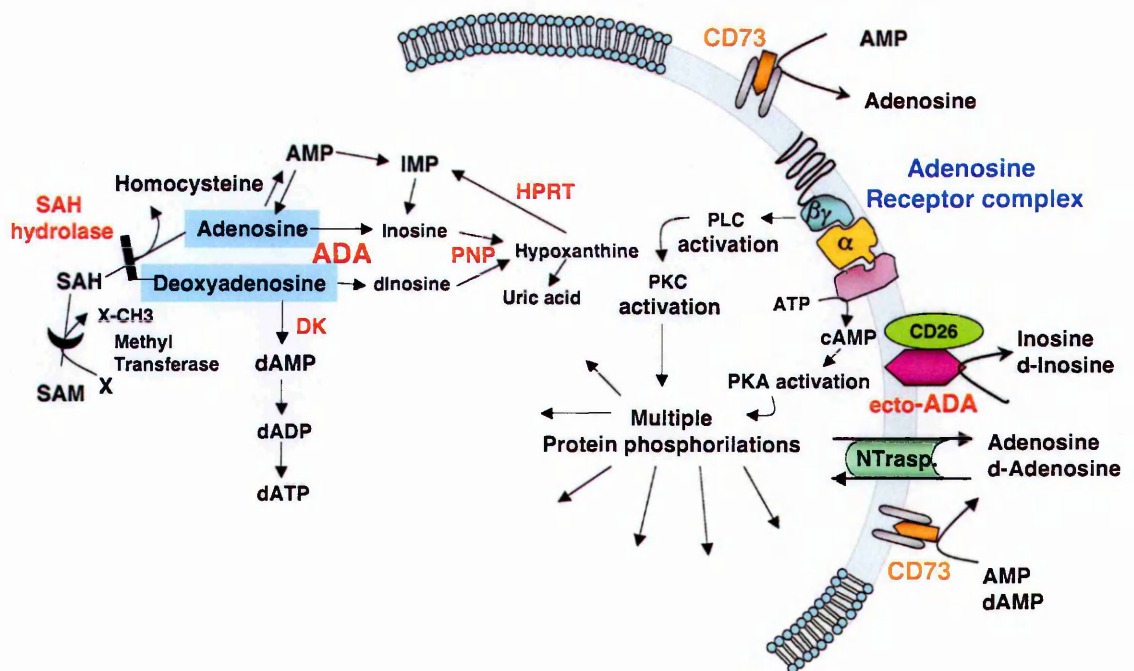
The cDNA length containing a coding region of 1.089 nucleotides predicts a polypeptide of 363 amino acids. However, the amino-terminal methionine is likely to be removed by post-translational processing to give rise a mature ADA peptide of 362 residues (Daddona et al., 1984). The crystal structure of the closely related mouse ADA shows a parallel  $\alpha/\beta$  barrel architecture with 8 central  $\beta$  strand and 8 peripheral  $\alpha$  helixes. A zinc ion essential for activity is situated in the active site pocket coordinated with three histidine and one aspartate residues (Illustration I) (Wilson et al., 1991).



**Illustration I. Three dimensional structure of ADA.** The image in the right panel presents the active site at the center, and polar and non-polar side chains are depicted in pink and yellow, respectively (From Franco R. et al., *Immun.Review*, 161, 1998)

In mammals, ADA exists in various molecular forms with different molecular weight. In cell extracts, ADA can be found in its monomeric 40kDa form and in molecular form of 200kDa or higher. This high molecular mass form results from the association of

ADA with an ADA-binding (or –complexing) protein (see next section for details) present on the cell surface. The small form represents the cytosolic enzyme of the purine salvage pathway, which catalyzes the irreversible deamination of Ado and dAdo into inosine and d-inosine, respectively (Illustration II). Most of Ado derived endogenously, from the normal intracellular breakdown of ATP and degradation of RNA, or taken up intracellularly, via an ubiquitously expressed nucleoside transporter, is normally phosphorylated rather than deaminated because the  $K_m$  for adenosine kinase is an order of magnitude lower than that for ADA (Simmonds et al., 1978). Unlike Ado, dAdo formed by DNA degradation is predominantly catabolized by ADA. Further mobilization of inosine nucleoside leads to hypoxanthine, which can be either enter a nonreversible pathway to uric acid or salvaged back into other mononucleosides.



**Illustration II. Role of ADA in the purine metabolism.**

HPRT, hypoxanthine phosphoribosyl transferase; NT, nucleoside transporter; PNP, purine nucleoside phosphorylase; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine, AK, adenosine kinase

In the absence of ADA, the presence of these alternative “bypass” pathways results in normal concentrations of the catabolic products of the enzyme reaction in patients with ADA-SCID. Conversely, the levels of ADA substrates, Ado and dAdo, not only are found in increased amounts in extracellular body fluids, but they also “spill over” into additional pathways normally only minimally utilized (Simmonds et al., 1978) (Mills et al., 1976) (Hirschhorn et al., 1982) thus contributing to the pathogenetic mechanisms of the disease (a detailed description is reported in section 1.6).

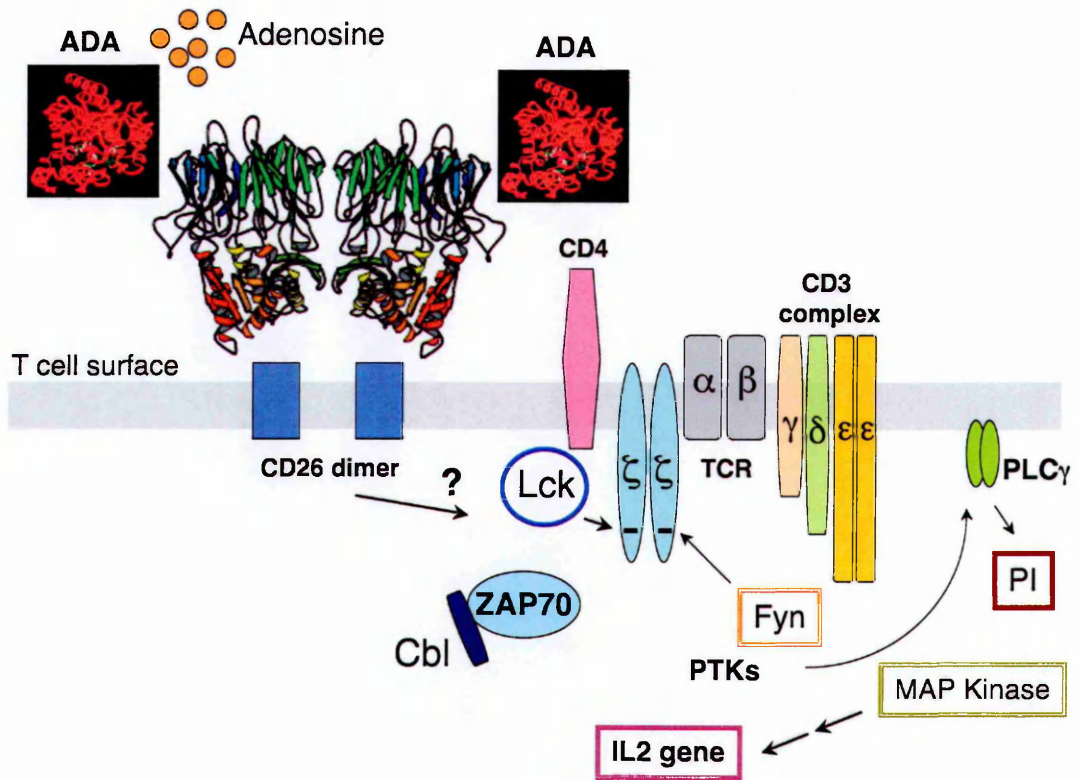
#### **1.4 Ecto-ADA: the cell-surface molecule and its anchoring proteins**

Although ADA is more than 90% intracellular, it can be found as ecto-enzyme associated to ADA-binding proteins at the membrane level in human but not murine cells (Dinjens et al., 1989). No differences in terms of catalytic activity and protein structure has been observed between the two isoforms, thus indicating the common function in deaminating Ado and dAdo. Two types of ADA-binding protein has been so far identified: CD26 and Adenosine Receptors.

##### **1.4.1 CD26**

The first ADA-complexing protein identified was CD26 (Kameoka et al., 1993). CD26 is a type II membrane sialoglycoprotein, consisting of two identical subunit of about 100 kDa, with a postproline dipeptidyl aminopeptidase activity (DPP IV) in the dimeric form. It has been demonstrated that CD26 does not participate in the transport of ADA toward the cell surface but anchors ADA after the protein has been released from homologous or heterologous cells (Dong and Morimoto, 1996). The CD26-binding site of human ADA has been localized to the peripheral alpha 2-helices (amino acids 126-143) and involved both charge and hydrophobic interaction (Jones and Thornton, 1996) highly conserved inter-species during the evolution. The significance of the selective advantage of this interaction is likely related to the additional control

provided over the extracellular concentrations of inhibitory purines. The enzymatic activities of ADA and CD26 are unaffected by their binding, indicating that this binding is independent from the catalytic functions of both the enzymes (De Meester et al., 1994). CD26 has a broad tissue distribution and among lymphoid cells, CD26 is expressed in medullar thymocytes (Dang et al., 1991) and at detectable level in peripheral lymphocytes, being its expression strongly upregulated following *in vivo* and *in vitro* activation (Fox et al., 1984), primarily in T cells. The significance of CD26 in T-cell function resides in its ability to modulate the activity of several chemokines (Oravecz et al., 1997) and to provide a costimulatory signal required for optimal T cell activation (Bristol et al., 1992) (Tanaka et al., 1993), which to a final extent results in upregulation of activation markers, increase cytokine secretion and proliferation, augment of T cell responses to foreign antigens, induction of differentiation into effector cells and enhanced helper function to B cells and cytotoxic T lymphocytes (Morimoto and Schlossman, 1998). The signal transduction machinery initiated by CD26 is not fully characterized. Cross-linking CD26 with a monoclonal antibody induces an increased tyrosine phosphorylation of a variety of protein similarly triggered by TCR-mediated stimulation (Illustration III), thus indicating a functional relationship between CD26 and TCR/CD3 complex (Hegen et al., 1997) (Mittrucker et al., 1995), but not their physical interaction.



**Illustration III. A model of CD26/DPP IV action at the T cell surface.** The ADA binding activity of CD26 contributes to the control of adenosine levels at the T-cell surface. The peptidase activity of CD26 requires dimerization but the dimerization-site is unknown. The CD26-triggered signal transduction pathway is CD3zeta chain dependent and includes phosphorylation of the PTKs p56 (Lck), p59 (Fyn) and zeta associated PTK of 70 000 kDa (ZAP-70), and of MAP kinase, Cbl, and phospholipase Cg, suggesting that such signals are transmitted to the nucleus via the TCR/CD3 pathway.

CD26 has been shown to coprecipitate with an isoform of CD45, expressed preferentially on CD45RO<sup>+</sup> memory T-cell subset (Torimoto et al., 1991). This interaction could account for the activation of protein tyrosine kinases and calcium mobilization upon anti-CD26 stimulation.

Several lines of evidence suggest that the binding of ecto-ADA to CD26 is a prerequisite for the exerted stimulatory properties on immune function, thus envisaging a possible important role of ecto-ADA as a co-stimulatory molecule in T cells.

### **1.4.2 Adenosine Receptors**

One of the substrates of ADA, Ado, is an autacoid that exerts multiple physiological actions in various systems (Tucker and Linden, 1993) (Fredholm and Dunwiddie, 1988) (Churchill and Bidani, 1982) (Merrill et al., 1997). Ado acts through specific receptors present on the surface of target cells, four of which have been cloned ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$ ; section 1.6.2). Adenosine receptor type 1 ( $A_1R$ ) (Ciruela et al., 1996) and 2B ( $A_{2BR}$ ) (Herrera et al., 2001) have been identified as the other ADA-binding proteins. It has been demonstrated that ADA and Ado receptors interact and that the proteins are functionally coupled. Indeed, ecto-ADA can regulate receptor engagement either by degrading extracellular Ado to inosine or modulating their binding affinity to other agonists (Herrera et al., 2001).

### **1.5 The enzymatic and extra-enzymatic role of ADA in the immune system**

ADA is present in all tissues but its levels vary widely and are controlled in a cell-specific as well as developmentally programmed manner, suggesting that ADA plays a more important role in some tissues or species than others. In humans, ADA activity is highest in the thymus followed by the peripheral lymphoid tissues, gastro-intestinal tract and possibly a portion of the cerebral cortex (Chinsky et al., 1990) (Witte et al., 1991). All of these tissues contain predominantly the small intracellular form of ADA but also detectable amounts of the ecto-ADA complex. In peripheral blood mononuclear cells, ADA is found as ecto-enzyme in the majority of monocytes and B cells and in some (10-20%) T cells (Aran et al., 1991). Other tissues including liver, lung and kidney have similar and lower activities of ADA, but greater than that found in erythrocytes. All of these tissues exhibit predominantly the large form of ADA. The enzyme activity is subjected to changes depending upon the state of cellular activation or differentiation. In the human thymus, ADA activity is highly elevated in cortical thymocytes and it is

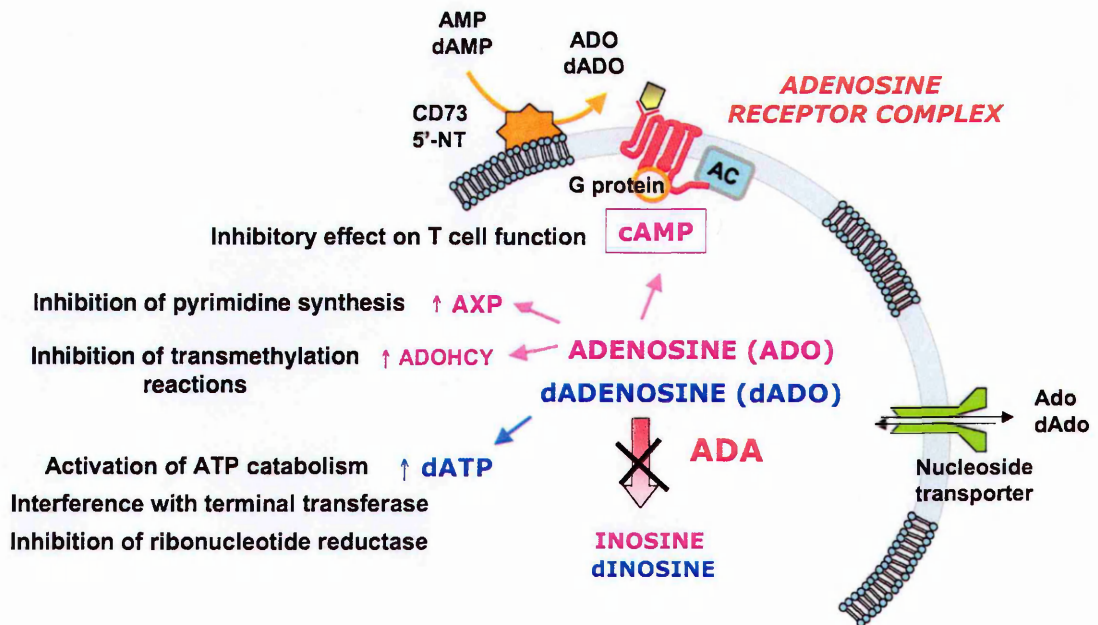


down regulated at the medullar stage (Chechik et al., 1981). In the mature lymphoid cells ADA activity is highest in T lymphocytes compared to B cells (Barton et al., 1979). In contrast with the total enzyme activity, the number of ADA molecules on the plasma membrane is much higher in B cells than in T cells (Aran et al., 1991). Until very recently, the role of ADA, irrespectively of its cellular localization, was thought to be the maintenance of non-toxic levels of purine metabolites. However, several experimental evidences have then suggested an extra-enzymatic role for the complexed form of ADA as co-stimulatory molecule, by virtue of its ability to physiologically interact with CD26. Firstly, exogenous ADA could enhance proliferation of CD4<sup>+</sup> cells induced by anti-CD3 even in presence of ADA inhibitors, indicating an enzyme-independent mechanism of ADA action (Martin et al., 1995). Secondly, incubation of T cells with exogenous ADA enhanced the increase in intracellular calcium induced by anti-CD3 mAbs, indicating that ADA initiated signaling events (Franco et al., 1998). Thirdly, anti-CD26 mAbs, that blocked ADA binding, costimulated anti-CD3-dependent T cell proliferation (De Meester et al., 1995) (Plana et al., 1991), suggesting that ligation of the ADA binding site on CD26 may be sufficient to trigger a signaling. The role of ecto-ADA in T-cell activation complements with its expression in resting or activated lymphocytes. Indeed, relatively low level of ecto-ADA can be detected in resting T cells whereas its expression, as for CD26, markedly increases upon activation (Franco et al., 1998). Based on the existence of signaling pathways in lymphocytes via the ADA/CD26 interaction and of other binding proteins, ecto-ADA has been demonstrated to mediate specific contacts between lymphocytes and dendritic cells at the level of immunological synapse, which finally resulted in potentiated T cell effector functions (Pacheco et al., 2005). This potential role of ecto-ADA in facilitate information exchange from cell to cell would be of relevance in the functionality and development of lymphoid tissues.



## 1.6 Biochemical basis of ADA-SCID

It is well established that the metabolic basis for ADA-deficient SCID are related to the physiological impact of the ADA substrates, Ado and dAdo. In the absence of ADA, these nucleosides are metabolized differently and have distinct biochemical action. dAdo behaves as a cytotoxic metabolite and is believed to provide the direct cause of the lymphocytes' depletion by intracellular poisoning. On the other hand, Ado may function as an extracellular signal transducer that mediates a variety of physiological effects by binding to G-protein coupled adenosine receptors present on the surface of target cells. The major toxic effects of Ado, dAdo and their nucleotides derives that have been considered as potential causes of the immune deficiency in ADA-SCID are summarized in Illustration IV.



**Illustration IV. Proposed pathogenetic mechanisms in ADA-SCID.**

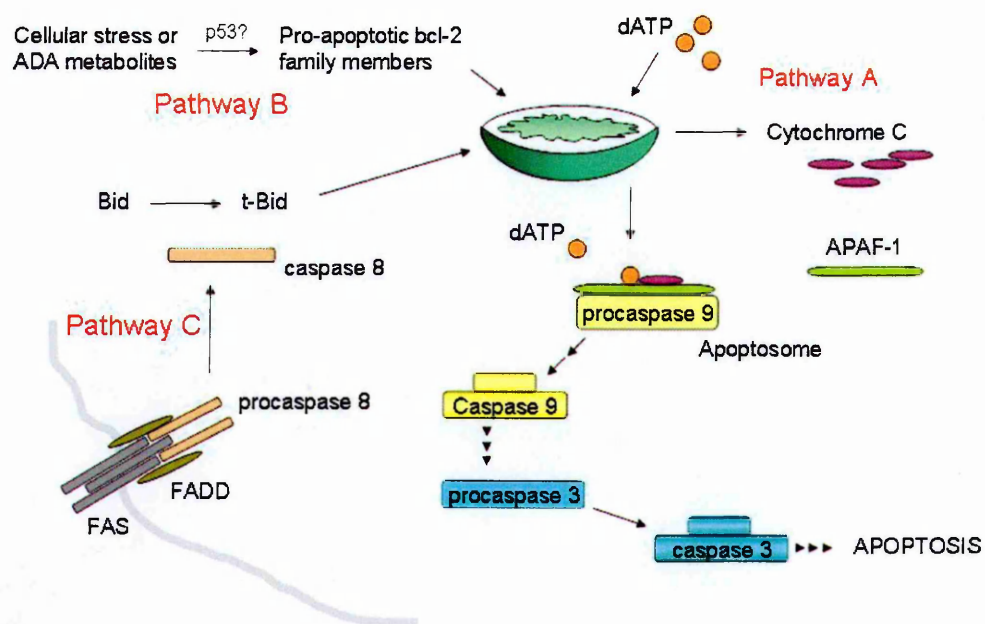
AXP, adenosine nucleotide derives; 5'-NT, ecto-5'-nucleotidase; AC, adenylyl cyclase; ADOHCY, S-adenosyl homocysteine.

However, the exact molecular mechanisms of lymphotoxicity are still elusive, and the relative contribution of intracellular toxicity vs signaling properties of extracellular adenosine remains incompletely understood. An important part of my PhD project has focused on the clarification of this aspect. This introduction presents the knowledge in the fields at the beginning of this project and the advancement, made in these years, which, together with our contribution, helped to elucidate the cellular and molecular basis of ADA-SCID pathology.

#### **1.6.1 Mechanisms of toxicity: dAdo.**

Although a limited knowledge exists regarding its own biological properties, the biochemical hallmarks of ADA deficiency are consistent with the general belief indicating dAdo as the primary cause of lymphotoxicity in ADA-SCID. Although dAdo is a weak substrate for Ado kinase and deoxycytidine kinase, in the absence of ADA these enzymes can phosphorylate dAdo. In turn, the resulting dATP pool expansion may interfere with a number of critical metabolic pathways. dATP is known to be a feedback inhibitor of ribonucleotide reductase, an enzyme required for normal DNA synthesis (Mann and Fox, 1986). In particular, it has been speculated that dNTP accumulation may preferentially exert its inhibitory effect in cells at the G1/S boundary, possibly preventing the association of the reductase with other complexing proteins required for initiation of replicative DNA synthesis (Waddell and Ullman, 1983). A reciprocal correlation has been established between the levels of ATP and dATP in red blood cells (RBC) of ADA-SCID patients (Simmonds et al., 1982), which manifested episodes of haemolytic anemia. The mechanism of dAdo-induced ATP catabolism was more readily explained by the ability of dATP to selectively activate the enzymes responsible for the adenine ribonucleotide catabolism (Bagnara and Hershfield, 1982). An additional mechanism was based upon *in vitro* findings demonstrating the inhibition of methyl-transfer reactions by suicide inactivation of SAH hydrolase consequent to

accumulating ADA substrates (Hershfield, 1979). Inhibition of SAH hydrolase has been also demonstrated *in vivo* in RBC of patients, but inhibition of specific methylation reactions has not been documented. In addition, SAH has been shown to act as a physiological modulator of APO-1/Fas-mediated cell death, suggesting another possible mechanism contributing to lymphocyte depletion in ADA-SCID (Ratter et al., 1996). The cause of lymphocytes depletion has been examined using foetal thymic organ cultures (FTOC) generated from gestational day -15 thymi (Thompson et al., 2000) (Van De Wiele et al., 2002). In the ADA<sup>-/-</sup> FTOC, thymocyte differentiation was blocked within the double negative (DN) stage. Introducing a Bcl-2 transgene or using FTOC from apaf<sup>-/-</sup> mice prevented the thymocyte loss induced by ADA inhibitor in ADA<sup>+/-</sup> FTOC, consistent with mitochondria-dependent apoptosis due to dATP accumulation. Indeed, the excess of dATP has been shown to promote the release of cytochrome c from mitochondria and facilitate its association with apaf-1 and procaspase 9 in the formation of apoptosome complex (Illustration V) (Purring-Koch and McLendon, 2000) (Yang and Cortopassi, 1998). dAdo was also reported to affect mature lymphocyte. Studies performed in human lymphocytes, in which deaminase has been chemically-inhibited, indicated that dAdo is able to interfere with early process of T cell activation, namely the hydrolysis of PIP<sub>2</sub> and the consequent IP<sub>3</sub> mediated mobilization of cytoplasmic free Ca<sup>2+</sup>, initiated by the interaction of PHA with its receptor (Scharenberg et al., 1989). The observed reduction in intracellular free Ca<sup>2+</sup>, which is vital to many signal transduction events, may account for the sensitivity of mitogen-stimulated lymphocytes to growth and IL-2 production inhibition by this toxic metabolite (Ruers et al., 1987).



**Illustration V. Postulated mechanism by which a lack of ADA can cause apoptosis in the thymus.** Pathway A: accumulated dATP causes release of cytochrome c from mitochondria, leading to formation of the apoptosome and initiation of the apoptotic cascade. Pathway B: abnormal levels of purine metabolites cause the induction of pro-apoptotic Bcl-2 family members, leading to cytochrome c release from mitochondria. Pathway C: abnormal levels of purine metabolites cause induction of death receptor signaling, leading to caspase 8 activation and cleavage of Bid. Truncated Bid then binds to pro-apoptotic Bcl-2 family members leading to release of cytochrome c from mitochondria.

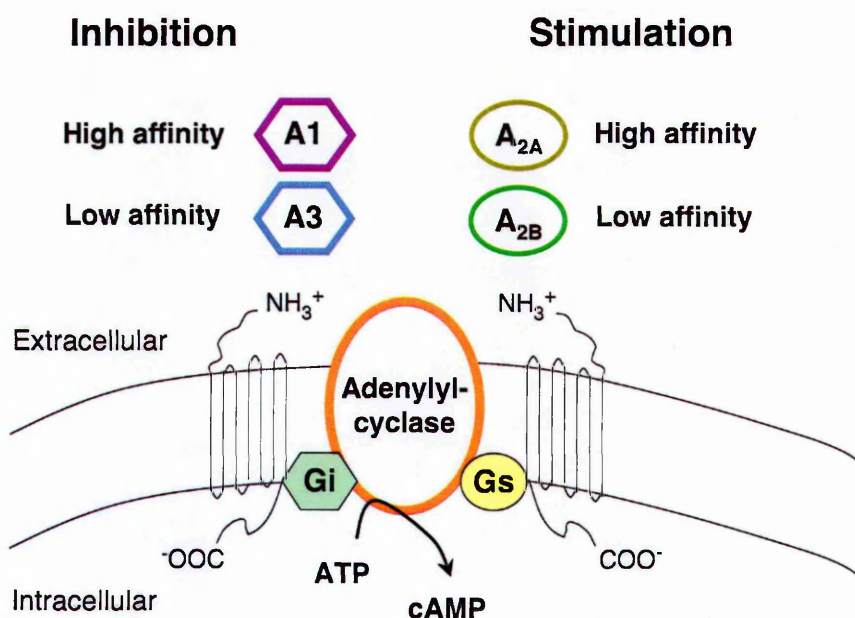
### 1.6.2 Mechanisms of toxicity: Ado.

The ability of Ado to cause depletion of cellular pyrimidine nucleotides has been one of the first mechanism proposed as the cause of lymphopenia in ADA deficiency (Green and Chan, 1973). Pyrimidine depletion *in vitro* requires the phosphorylation of Ado, and appears to be dependent by inhibition of phosphoribosylpyrophosphatase (PRPP) synthetase. However, neither exogenously added uridine nor inhibited adenosine kinase were able to prevent the Ado-mediated block of proliferative responses of human peripheral blood lymphocytes (Carson and Seegmiller, 1976) (Hershfield et al., 1977). Moreover, normal levels of UTP and CTP were found in the

body fluids of one ADA-deficient patient (Mills et al., 1979), thus the occurrence of pyrimidine starvation *in vivo* in ADA-SCID is still to be demonstrated.

Alternatively, Ado may initiate an aberrant extracellular signaling by binding to G-protein coupled adenosine receptors present on the surface of target cells (Sitkovsky et al., 2004). Physiologically, Ado-mediated triggering of these receptors can promote a fine-tuning of the inflammatory responses, but in the context of defective ADA-metabolizing enzyme, where the extracellular levels of adenosine would be likely dramatically increased, this regulation may be overstated, leading to immune defect.

To date, four subtypes have been identified: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> (Fredholm et al., 2001).



**Illustration VI. Classification of adenosine receptors and their coupling to the enzyme adenylyl cyclase.** Adenosine receptors belong to the family of heptahelical transmembrane G-protein-coupled receptors. Extracellularly orientated adenosine receptors bind adenosine with high and low affinity and can either stimulate or inhibit the enzyme adenylyl cyclase. The adenylyl cyclase consist of two regulatory and two catalytic units and generate from ATP the potent second messenger, cAMP.

Their cloning helped to prove that high-affinity  $A_{2A}$  and low-affinity  $A_{2B}$  receptors activate adenylyl cyclase, whereas high-affinity  $A_1$  and low-affinity  $A_3$  inhibit it (Illustration VI). Accordingly, when immune cells acquire the expression of all types of these receptors, they will be recruited in a stepwise manner with Gi-coupled  $A_1$  receptors activated first at very low Ado levels, followed by the stimulation of Gs-coupled  $A_{2A}$  and  $A_{2B}$  receptors, and finally by Gi-coupled  $A_3$  receptors (Fredholm et al., 2001). When activated pharmacologically, cAMP-elevating  $A_{2A}$  and  $A_{2B}$  receptors inhibit inflammatory immune responses (Sitkovsky, 2003) (Linden, 2001). Activation of  $A_3$  receptors exerts anti-inflammatory effects by still unknown mechanisms (Salvatore et al., 2000). Very low concentrations of Ado can bind to and activate  $A_1$  receptors that can inhibit the adenylyl cyclase-activating  $A_{2A}$  and  $A_{2B}$  receptors. Because higher levels of Ado can overcome the inhibition mediated by  $A_1$  receptors,  $A_1$  receptors are thought to exert a tonic inhibitory effect on  $A_2$  receptor functions.

The effects of Ado-mediated signaling on immune cell function will be discussed hereinafter, in the view of their possible involvement in the main cellular defects observed in ADA-SCID.

#### **1.6.2.1 Thymocytes**

The thymic environment is normally conducive to Ado-mediated signaling, as significant amount of Ado is generated through physiological mechanisms of lymphocyte selection. Accumulated Ado can induce apoptosis in mouse or human thymocytes (McConkey et al., 1994) (Szondy, 1994), mostly via adenosine  $A_{2A}$  receptors. The signaling pathway involves adenylyl cyclase and the pro-apoptotic protein Bim, and is negatively regulated by Nur77 transcription factor (Kiss et al., 2006). The ADA-SCID knock-out mouse model, retaining many of the features associated with ADA-deficiency in humans, was generated by two-stage genetic

engineering (Blackburn et al., 1998). An abundance of apoptosis was observed in thymi of ADA<sup>-/-</sup> mice compared to control animals (Apasov et al., 2001), localized predominantly to the cortical-medullary boundary, where CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes should accumulate (Apasov et al., 2001). A direct apoptotic effect on this specific thymocyte subset by extracellular Ado was demonstrated using A<sub>2A</sub> receptors knock-out mouse (Apasov et al., 2000), thus suggesting that thymocytes depletion in ADA-SCID is likely due to aberrant engagement of A<sub>2A</sub> receptor-mediated signaling. Moreover, reduced phosphorylation of CD3  $\zeta$  chain and inhibition of Ca<sup>2+</sup> mobilization has been described *in vivo* and *in vitro* in ADA<sup>-/-</sup> thymocytes (Apasov et al., 2001) and attributed to Ado. Therefore, two alternative or simultaneously operating mechanisms could account for T cell depletion in ADA-SCID: a) a direct apoptotic effect and b) inhibition of TCR signaling and, hence, inhibition/block of TCR-driven process of T-cell selection.

#### **1.6.2.2 Mature T lymphocytes**

Mature CD4- and CD8-positive T cells from ADA<sup>-/-</sup> mice have decreased TCR-triggered activation (low CD25 and CD69 marker expression) *in vivo* and *in vitro* (Apasov et al., 2001). This inhibitory effect is more readily explained by Ado-mediated signaling through A<sub>2A</sub> receptor, the major adenosine receptor subtype expressed in murine as well as human lymphocytes (Koshiba et al., 1999) (Thiel et al., 2003). Indeed, although the biochemical mechanisms potentially involved have been incompletely demonstrated in ADA-SCID, studies carried out with artificial system, using ADA-inhibitory drugs or selective A<sub>2A</sub> receptors agonists/antagonists, showed that the pharmacologic increase of intracellular cAMP levels interferes with the proximal biochemical events following T-cell receptor-triggering, leading to inhibition of interleukin-2 receptor  $\alpha$  chain (CD25) upregulation (Huang et al., 1997). This event associated with decreased tyrosine phosphorylation of STAT-5 proteins, due to

activation of SHP-2 tyrosine phosphatase (Zhang et al., 2004), and TCR-triggered proliferation. In activated CD4<sup>+</sup> T cells Ado had a broad inhibitory effect on secretion of IL-2, INF- $\gamma$ , TNF- $\alpha$  and IL-4 cytokines (Lappas et al., 2005) (Erdmann et al., 2005), whereas in resting lymphocytes it induced upregulation of CD152, CTLA-4, normally involved in the termination of immune response (Vendetti et al., 2002). In addition to defective cytokine production and Ag-driven proliferation (Erdmann et al., 2005), CD8<sup>+</sup> T cells displayed reduced granule exocytosis and FasL mRNA upregulation upon A<sub>2A</sub> receptors ligation (Koshiba et al., 1997), which may account for the inability to clear viral infection *in vivo*. The A<sub>2B</sub> and A<sub>3</sub> receptor types are upregulated, as the A<sub>2A</sub>, in activated lymphocytes (Mirabet et al., 1999) (Gessi et al., 2004). However, while A<sub>2B</sub> increases its expression predominantly in CD8<sup>+</sup> cells, A<sub>3</sub> is upregulated mainly exclusively in CD4<sup>+</sup> cell subset, thus leading to selective regulation of both cytotoxic CD8 and helper CD4 T cell activities. Overall, these evidences strongly suggest that the extracellular-related mechanisms of toxicity might be relevant as the intracellular poisoning in the pathophysiology of the disorder. In this view, the engagement of an aberrant adenosine receptor signaling may offer a better comprehension for the autoimmune manifestations observed in ADA-SCID patients (Kohn DB, 1996) (Ozsahin et al., 1997), because of their cell-specific expression and regulation.

### **1.6.2.3 B lymphocytes**

With respect to T lymphocytes, limited knowledge is available about the effects exerted by Ado on B-cell function. However, recent studies have defined downstream consequences of adenosine receptors ligation on the response of B cells to antigen-receptor engagement. Minguet et al. reported that exogenous Ado inhibits NF- $\kappa$ B activation in primary murine B cells stimulated via the BCR or by LPS via TLR4 (Minguet et al., 2005). The effect of Ado on NF- $\kappa$ B activation in B cells was associated to inhibition of BCR-induced I $\kappa$ B phosphorylation. Therefore, Ado may drive BCR-



stimulated B cells towards an anergic rather than an immunogenic response. Such a mechanism might contribute to B-cell dysfunction in ADA deficiency, and may better explain the defective B-cell activation and proliferation observed in ADA<sup>-/-</sup> mice.

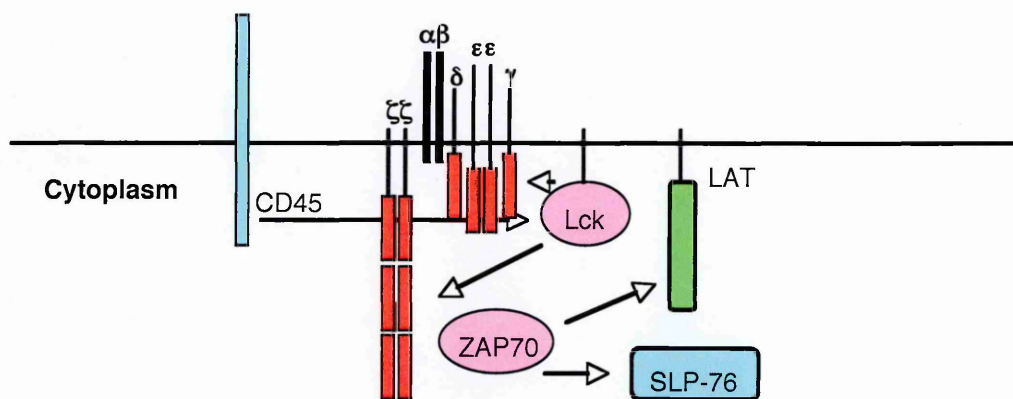
### **1.7 TCR signaling: an overview**

The detection of antigens by T cells is achieved through recognition by the TCR of antigenic peptides presented in association to MHC molecules. The final outcome of this recognition can be either full activation and acquisition of effector functions or establishment of anergy. The current model of T cell activation proposes that at least two signals must be provided to a naïve T cell to be fully activated and to avoid anergy (Linsley and Ledbetter, 1993; Schwartz, 2003). The first signal is necessarily delivered through the TCR, while the second signal, generally referred to as costimulation, is provided by receptors expressed by APCs or by soluble factors. The main molecule involved in the delivering of “signal two” is CD28, whose natural ligand (B7.1/2) is highly expressed by activated/mature APC (Acuto and Michel, 2003). Engagement of the TCR elicits a cascade of signaling events, which leads to transcriptional activation of multiple genes involved in T cell proliferation and differentiation, and to cytoskeletal rearrangements (van Leeuwen and Samelson, 1999). The main molecular events downstream of TCR and CD28 triggering will be presented here.

#### **1.7.1 TCR signaling: proximal events**

The first event upon TCR triggering is the phosphorylation of different tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3- $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  invariant chains of the TCR. This phosphorylation is exerted by Src kinases (mainly Lck) and regulated by the phosphatase CD45 (Zamoyska et al., 2003) (Palacios and Weiss, 2004). Phosphorylated ITAMs mediate the recruitment and activation of the Syk kinase ZAP-70 (Neumeister et al., 1995). Full phosphorylation of the CD3- $\zeta$  is

essential for productive T cell activation (Sloan-Lancaster et al., 1994) and is achieved with the help of CD4/CD8 co-receptors, which are constitutively associated to Lck. ZAP-70 is a central kinase as it phosphorylates a number of molecules including the adaptor molecules LAT (linker of activated T cells), which is constitutively rafts-associated (Zhang et al., 1998) and SLP-76. LAT-SLP-76 complex is a fundamental organizer of TCR signaling (Illustration VII) (Myung et al., 2000).

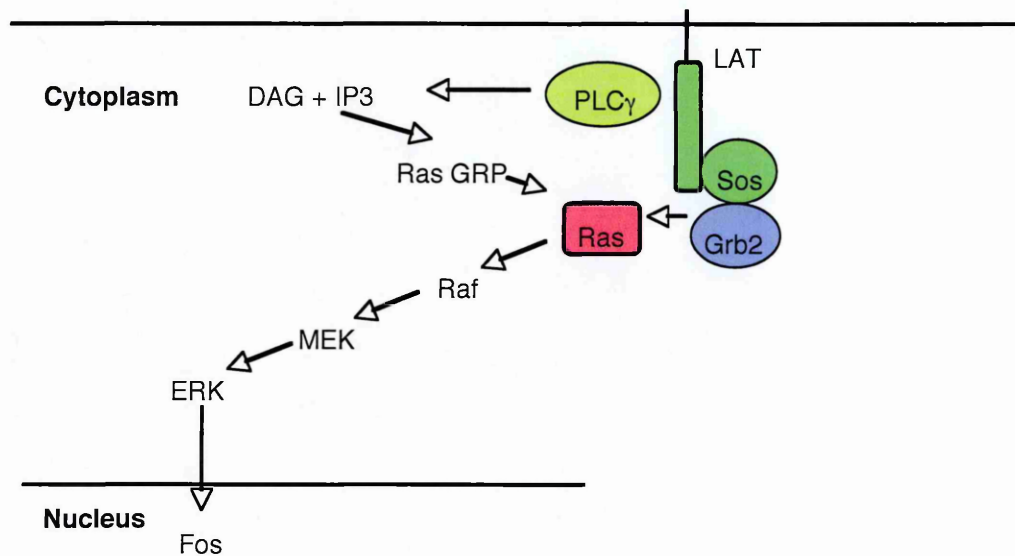


**Illustration VII.**

**a. ERKs activation**

One crucial function of LAT is coupling the TCR to the Ras pathway. Indeed, phosphorylated LAT recruits the adaptor protein Grb2, which associates with the Ras guanine nucleotide exchange (GEF) protein, Sos. Sos/Grb2 can activate the small G protein Ras, although an alternative pathway of Ras activation has also been described, involving the guanyl nucleotide releasing protein GRP, which can be recruited by DAG (see after) (Cantrell, 2003b). Activation of Ras is a crucial event, as it initiates the activation of the Raf-1/MEK signaling pathway, which in turn leads to activation of RSK2 and ERKs. ERK1/2 (also called p44/42 MAPKs) controls T cell cycle

progression and the transcription of the Fos gene (Illustration VIII) (Hunter and Karin, 1992). Fos gene transcription is essential for the activation of the AP-1 complex, which is required for IL-2 gene transcription (see section g).



**Illustration VIII**

### c. Inositol lipid metabolism (I): from PLCγ to activation of NFAT

In addition, LAT, together with SLP-76 and the adaptor Gads, mediates the recruitment of PLCγ, a key molecule of the inositol lipid metabolism. PLCγ activation requires the activity of Lck, Zap-70 and of the Tec kinases (see section e). The products of PLCγ enzymatic activity are inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 is responsible for release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (ER), which then mediates the opening of  $\text{Ca}^{2+}$  channels ( $\text{Ca}^{2+}$  release activated channels: CRAC) on the plasma membrane (Lewis, 2001).  $\text{Ca}^{2+}$  increase induces the activation of several calmodulin-dependent enzymes, including calcineurin and CAMKII. Calcineurin has a crucial role as it dephosphorylates multiple phosphoserine residues on

the transcription factors NFAT. Phosphorylated NFAT is retained in the cytoplasm in resting cells, and upon dephosphorylation it translocates to the nucleus, where it mediates the activation of many cytokine genes, including IL-2, IL-4, IFN- $\gamma$ , IL-3, GM-CSF, IL-10 (Hogan et al., 2003). Five NFAT family members have been identified, which are named NFAT-1-2-3-4 (also called NFATc2-1-4-3) and NFAT-5 (also called TonEBP), which is regulated in a different way.

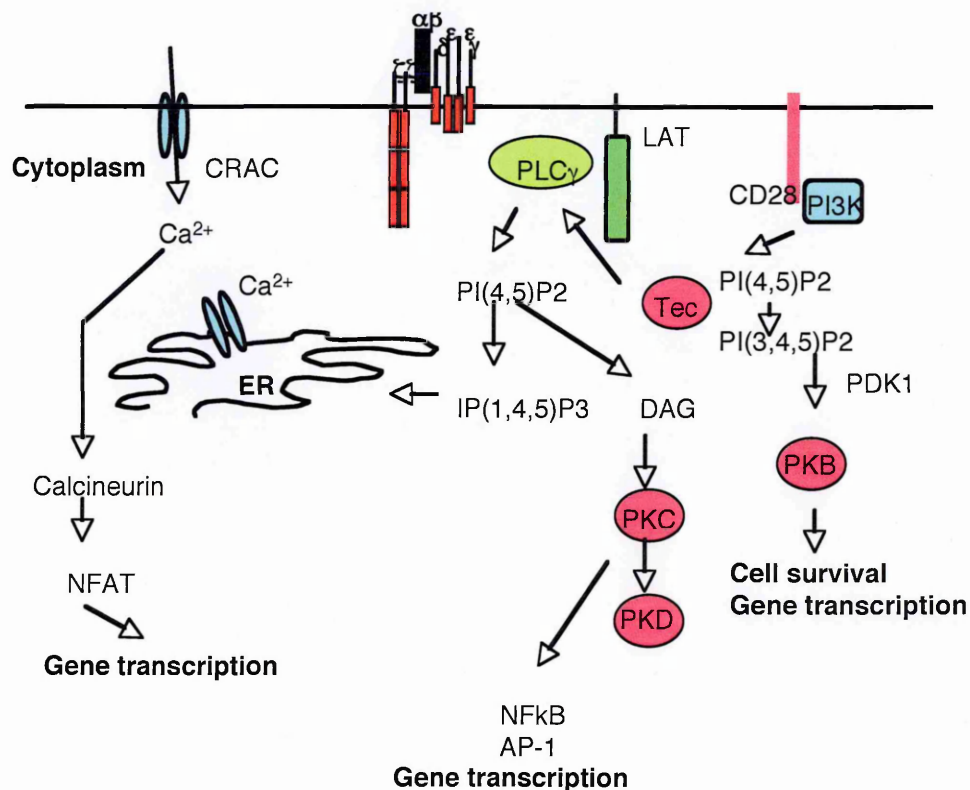
**d. Inositol lipid metabolism (II): from PLC $\gamma$  to activation of PKC**

Production of DAG is critical for the activation of the serine/threonine kinases of the PKC family and also to link lipid metabolism to the Ras pathway (Ebinu et al., 2000). PKCs can be divided into two groups according to their dependence on Ca<sup>2+</sup> (Mellor and Parker, 1998). In T cells the more relevant form of PKC is the Ca<sup>2+</sup>-independent PKC $\theta$ . The importance of PKC $\theta$  in T cell activation has been highlighted in the last years. This kinase is specifically expressed in T cells (and in skeletal muscle). Upon TCR triggering, PKC $\theta$  translocates to the immune synapse at the levels of lipid rafts (Bi et al., 2001) and it positively regulates IL-2 gene transcription, likely by modulating the activity of the transcription factors AP-1 and NF $\kappa$ B (Bi et al., 2001; Lin et al., 2000; Werlen et al., 1998).

**e. Inositol lipid metabolism (III): PI3K signaling**

A second pathway of inositol lipid metabolism activated by TCR and CD28 involves the activation of PI3K (Cantrell, 2001). PI3K can be divided into three classes. Class I PI3Ks are generally coupled to extracellular stimuli. In T cells, PI3K, which can be also activated through CD28, independently on TCR triggering (Ward et al., 1993), generates PI(3,4,5)P3, starting from PI(4,5)P2. PI(3,4,5)P3 is then metabolized to produce PI(3,4)P2. Generation of PIP3 and PI(3,4)P2 is crucial as these lipids mediate the recruitment of several proteins containing pleckstrin homology (PH) domains, including the Tec kinases (Finkelstein and Schwartzberg, 2004), the phosphoinositide

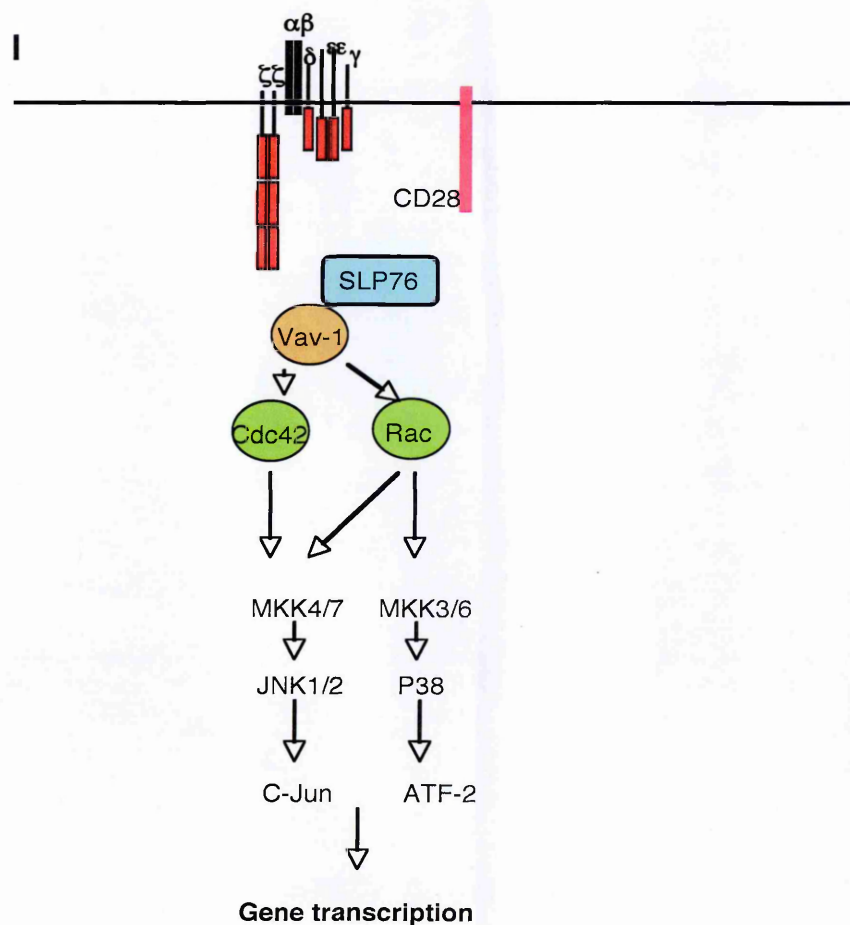
dependent protein kinase (PDK) 1, the protein kinase B (PKB/AKT) (Cantrell, 2001) and, possibly, Vav-1, which is the guanine nucleotide exchange proteins (GEFs) for Rho, Rac and Cdc42 . PDK1 targets different kinases, including p70S6K1, GSK3 and PKB itself, thus regulating a variety of processes including T cell size, growth, survival, glucose metabolism and NFAT localization (Cantrell, 2003a) (Patra et al., 2004). Tec kinases belong to a large family of non-receptor tyrosine kinases. T cells mainly express Itk and Rtk Tec kinases (Schwartzberg et al., 2005). Besides being required for full PLC $\gamma$  activation, Tec kinases exert an important role in coupling TCR/CD28 triggering to actin cytoskeleton rearrangement, by association and functional interaction with Vav-1 and WASP. A schematic representation of inositol lipid metabolism is depicted in Illustration IX.



**Illustration IX.**

**f. Activation of JNK and p38 MAPKs**

Activation of Rho GTPases links TCR/CD28 triggering to the activation of the MAPKs JNK1/2 and p38 (Illustration X) (Davis, 2000; Dong et al., 2002). Both p38 and JNK are involved in the transcriptional activation of IL-2 gene, the first by phosphorylation of ATF2 transcription factor, the second by phosphorylation of the c-Jun transcription factor, which, together with Fos, forms the AP-1 complex (Illustration XII) (Macian et al., 2001). In addition, JNK has been shown to stabilize IL-2 mRNA (Chen et al., 2000).



**Illustration X.**

**g. AP-1, NFκB and CREB**

AP-1 complex is formed by Fos family members (c-Fos, FosB, Fra-1 and Fra-2) and by Jun family members (c-Jun, JunB and JunD). AP-1 dimer binds DNA either alone, or together with NFAT (Karin et al., 1997). During T cell activation, AP-1 binds and positively regulates the IL-2 promoter. NFκB comprises a family of factors which acts as dimers. NFκB is retained in the cytoplasm of resting T cells through the binding to the inhibitors of κB (IκBs). Phosphorylation of IκBs by IκB kinases (IKKs) determines their degradation through the ubiquitin-proteasome pathway. PKCθ is involved in NFκB activation, possibly by direct phosphorylation of IKKβ (Lin et al., 2000). Importantly, NFκB activity is also regulated by direct phosphorylation of its transactivating subunits p65, c-Rel and relB (Weil and Israel, 2004). NFκB dimers bind to IL-2 promoter, contributing to IL-2 gene transcription. The transcriptional activity of CREB is regulated by a RSK2-mediated single phosphorylation on Ser-133 that leads to complex formation with the coactivator, CBP and binding to CRE elements (Brindle et al., 1993). In T cells, CRE elements can be found in TCR and CD3 genes as well as in other genes involved in lymphocytes activation, including cytokine genes (Barton et al., 1996). A schematic representation of IL-2 gene transcription is depicted in Illustration XI.

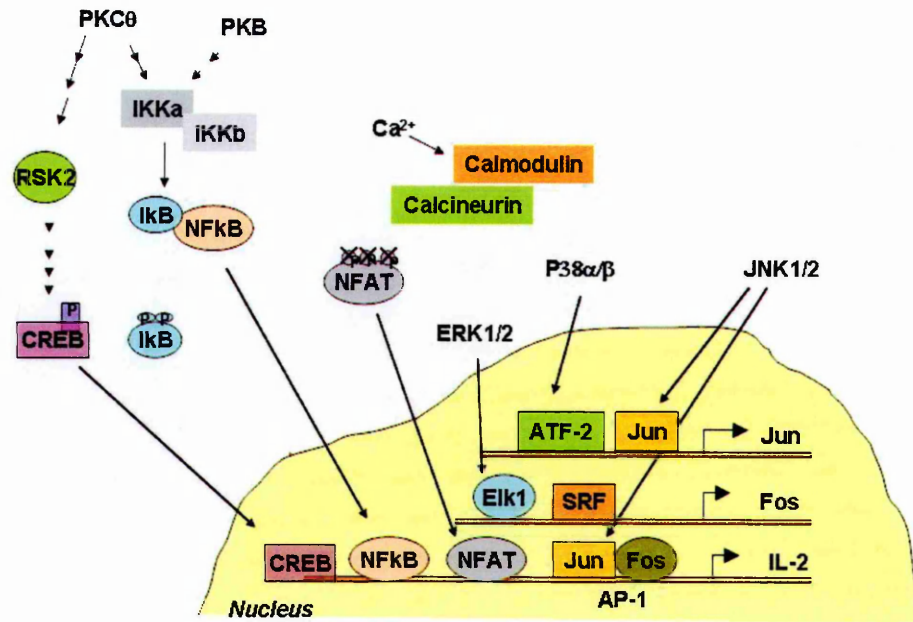


Illustration XI.

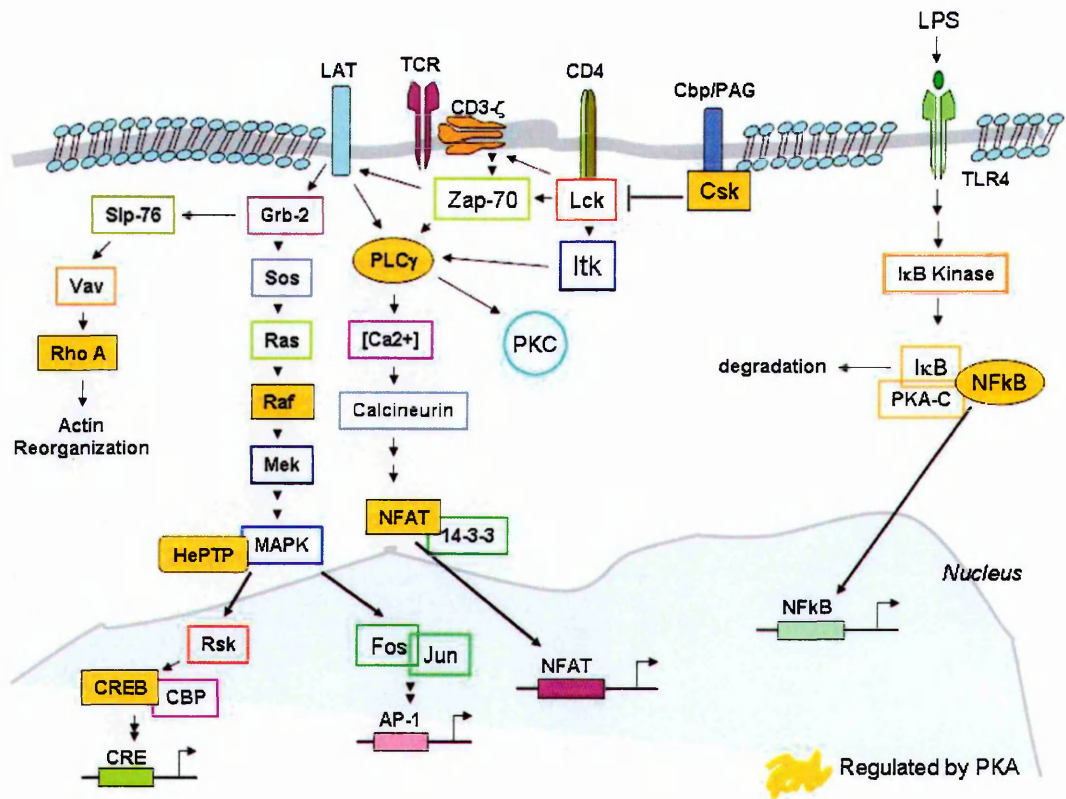
### 1.7.2 Regulation of TCR signaling by cAMP/PKA I pathway

Control and fine-tuning of the proximal TCR signaling are required both for effective activation and to avoid inappropriate or exaggerated immune activation and autoimmunity. In this regard, inhibitory signaling mechanisms interact with the activated pathways as means of modulating the immune responses. The role of cAMP and cAMP-dependent protein kinases (protein kinase A, PKA) in such modulation of immune function is discussed here.

Stimulation of the TCR alone leads to a transient rise in cAMP (Ledbetter, JI 1986) levels, generated by recruitments of Gs and dissociation of Gi proteins in lipid rafts. Increased cAMP serves to down-modulate TCR-triggered signal transduction and prevent full T cell responses, as a consequence TCR-mediated cAMP production must



be regulated for T cell activation to occur. Recently, it has been shown that stimulation of the coreceptor CD28 markedly potentiates the recruitment of a  $\beta$ -arrestin/PDE4 complex into rafts upon TCR activation (Abrahamsen et al., 2004). Delivery of active PDE4 enhanced cAMP degradation, thereby amplifying T cell activation. With few exceptions, the effects of cAMP are mediated by cAMP-dependent protein kinase A type I (PKAI) (Torgersen et al., 2002). This isoenzyme is thought to play a major role in cAMP-mediated effects in T lymphocytes, because it redistributes and colocalizes with the capped TCR (Vang et al., 2001). PKA regulates immune function at multiple levels (Illustration XII). PKA consensus phosphorylation sites have been identified in NFAT, NF- $\kappa$ B and CREB transcription factors, indicating that they can be regulated by PKA activity. Phosphorylation of NFAT by PKA occurs at conserved sites adjacent to a nuclear localization sequence that regulates the association between NFAT and 14-3-3 protein (Chow and Davis, 2000). Dephosphorylation by Calcineurin results in release of NFAT from 14-3-3 and its nuclear translocation. A proportion of PKA catalytic subunit (PKA-C) binds to I $\kappa$ B and associates with NF- $\kappa$ B-I $\kappa$ B complex (Zhong et al., 1997). PKA-C is kept in inactive state until I $\kappa$ B is degraded and the active PKA-C is released.



**Illustration XII. Intracellular signaling is regulated by PKA at multiple levels.** Several targets for PKA phosphorylation have been localized that can modulate the immune response. At the transcriptional level, this includes CREB, NFAT and NF-κB (p56Rel). Further upstream targets like HePTP, Ras, Raf, MEK and MAPK provide means for PKA to regulate MAPK pathway differentially, while PKA phosphorylation of PLCγ can modulate signaling through phosphatidylinositols. Finally, PKA substrates like Csk and RhoA make PKA able to regulate proximal T-cell signaling and cytoskeletal processes important in immune activation.

Active PKA-C will then phosphorylate the p65 Rel subunit of NF-κB leading to increased transactivating activity of NF-κB, possibly by increasing binding of the coactivator complex CBP/p300 to p65. Although activation of CREB after TCR stimulation seems to be independent of PKA and rather involves RSK2 pathway, the ability of PKA to phosphorylate Ser-133 clearly opens for similar effects of stimuli activating PKA (Gonzalez and Montminy, 1989). PKA-mediated modulation of protein in the MAP kinase pathway has also been identified. The Ras-Raf interaction may be attenuated by PKA phosphorylation of Ser-43 in the Ras-interacting domain of Raf-1 (Wu et al., 1993). Further downstream, PKA phosphorylation of Ser-23 in the small

haematopoietic phosphatase HePTP leads to dissociation of MAPKs from HePTP and allows activation by Mek (Saxena et al., 1999). Treatment of T cells with cAMP-elevating agents resulted in PKA phosphorylation of PLC- $\gamma$  and suppression of  $\text{Ca}^{2+}$  mobilization and phosphatidylinositol hydrolysis upon T cell activation (Alava et al., 1992). Interestingly, cAMP causes morphological changes in different cell types including lymphocytes, which appears to involve PKA-mediated phosphorylation and regulation of RhoA (Lang et al., 1996) (Laudanna et al., 1997). Since Rho proteins family are key players in the reorganization of actin cytoskeleton during immune activation, it appears clear the significance of PKA activity in the formation of the immunological synapse. Finally, PKA-mediated inhibitory pathways can be assembled even at the site of T cell activation to provide precise temporal and spatial modulation of proximal TCR signaling. Indeed, PKA phosphorylates/activates Csk, which subsequently inhibits Lck, thereby attenuating T cell activation.

## **1.8 ADA-SCID: pathology**

### **1.8.1 Diagnosis**

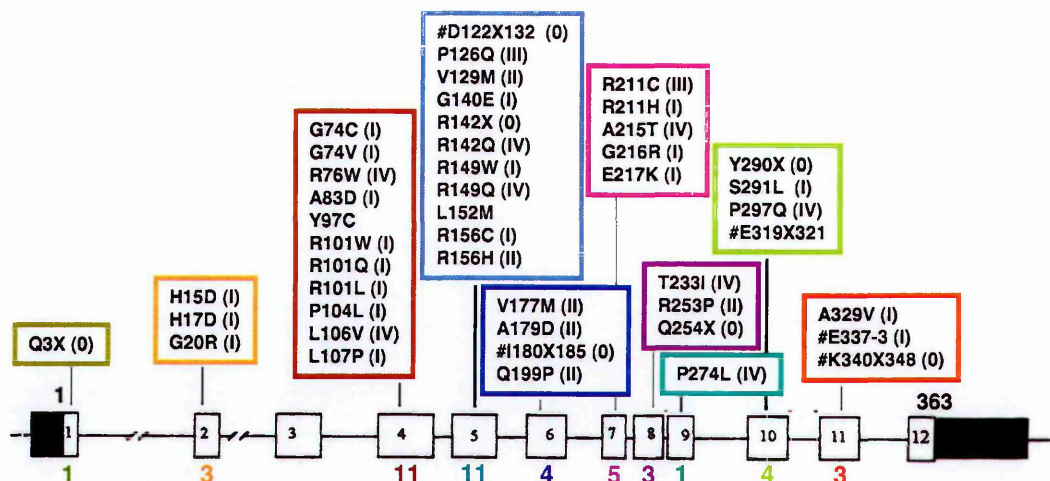
In the absence of ADA, the purine metabolites Ado, dAdo and adenine deoxyribonucleotides (dAXP) accumulate at high levels in plasma, red blood cells, and tissues. These metabolic hallmarks of ADA deficiency provide the biochemical diagnosis of ADA SCID, which is essentially based on ADA activity and dAXP concentrations in erythrocytes, measured by a capillary electrophoretic separation techniques. A dramatic reduction of ADA activity ( $56.9 \pm 48.3$  nmol/s per liter of packed cells vs  $5619 \pm 2584$  nmol/s/L in healthy individuals) and high dAXP concentrations in erythrocytes (300-450  $\mu\text{mol/L}$  vs undetectable level in healthy individuals) represent a universal finding for homozygous ADA deficiency (Carlucci et al., 2003). Heterozygosity for the defect can be difficult to detect, since many heterozygote carriers

have ADA levels at the lower limit of the normal range (Morgan et al., 1987). Later onset patients may retain 2-5% of normal activity, with the highest residual activity reported in case the adult onset of the disease. An additional group of individuals have been described who lack ADA in erythrocytes but express 5-80% of normal activity in lymphoid cells. It has been referred to these individuals as “partially” ADA deficient. Prenatal diagnosis is possible using chorionic villus sampling (Perignon et al., 1987), fetal blood (Morgan et al., 1987) and cultured amniotic fluid cells. Since ADA activity could be considered a useful prognostic indicator even in these cell type, its determination may help in the clinical management of the newborn patients and in the evaluation of the best therapeutic option.

### **1.8.2 Genotype-phenotype correlation**

More than 50 unique *ADA* gene mutations were identified from 59 unrelated families and collected in an ADA database (<http://www.uta.fi/imt/bioinfo/ADAbase/>). These mutations are scattered throughout the entire length of the *ADA* gene, although some hotspots have been identified (Arredondo-Vega et al., 1998) (Hirschhorn et al., 1990), landed in critical region of the molecule important for the substrate binding or the catalytic activity (exon 4 and 7; Illustration XIII). Mutations in ADA gene are consistent with alterations in ADA activity, enzyme stability and survival (Hirschhorn, 1999).

Over two-third of the patients are genetic compounds, carrying two different allelic mutations, with the remainder homozygous for the same mutation. Many patients with homozygous mutations are from populations with high frequency of inbreeding.



**Illustration XIII. Mutations causing ADA deficiency.** Three large 5' genomic deletions are not shown (two of 3.25 kb that include the promoter and Exon 1, and a >30 kb deletion that includes exons 1-5). For exonic mutations, the number in parentheses after the mutation name denotes the effect on ADA activity, as defined in *Arredondo-Vega et al., 1998*: deletion and nonsense mutations are designated "0", and missense mutations "I", "II", "III", or "IV" based on increasing ADA activity derived from constitutively expressing an ADA cDNA carrying the mutation in an ADA deletion strain of *E. coli*. Patients with genotypes composed only of alleles from groups "0" or "I" were very likely to have SCID, whereas those with at least one allele from groups "II" or "III" were very likely to have a milder "delayed onset" or "late/adult onset" phenotype; individuals who possessed one group "IV" allele were healthy and had "partial ADA deficiency" (*Arredondo-Vega et al., 1998*). Mutations designated IV were also associated with "partial" deficiency and found to provide substantial residual activity by semiquantitative methods (*Hirschhorn et al., 1990*).

Studies performed on large cohorts of patients allowed to highlight a good correlation between genotype and both clinical and metabolic phenotype. Thus, mutations that express residual ADA activity have been associated with milder form of disease. Indeed, certain missense and some splicing mutations found in healthy individual with "partial" deficiency or in immunodeficient patients with delayed/late onset phenotypes, have not been retrieved in patients with SCID, the most common early-onset phenotype (*Hirschhorn, 1995*).

### 1.8.3 Clinical aspect of immunodeficiency

The majority of ADA-SCID patients are diagnosed in the first year of life (early onset) and rarely survive beyond 1 to 2 years unless a therapeutic intervention leading to restored immune function is provided (Parkman et al., 1975). Thymic hypoplasia, broad lymphopenia, absence of humoral- and cellular-mediated immunity, recurrent infections and failure to thrive are consistent with the early onset forms of ADA-deficiency (Giblett et al., 1972) (Buckley et al., 1997). About 20% of ADA-SCID cases occur later in childhood (delayed) or beyond (late/adult onset). These forms show progressive immunological and clinical deterioration, often associated with autoimmune manifestations (Parkman et al., 1975) (Aiuti et al., 2003).

#### 1.8.3.1 Infections

The vast majority of early onset ADA-SCID patients have a history of recurrent infections, which can be life-threatening. Initially, infections involve areas with the greatest exposure to organism, such as skin, gastrointestinal and respiratory systems. Infectious agents include bacteria, fungi, viruses and protozoa (Meuwissen et al., 1975). *Pneumocystis carinii* pneumonia and giant-cell pneumonia occur frequently. Candidiasis is almost invariably present, causing both “rash” and more extensive infections involving skin, oral and esophageal mucosa. Diarrhea, which is generally secondary to abnormal intestinal flora, is a common feature and may seriously compromise nutrition. Later-onset forms are characterized by recurrent sinopulmonary bacterial, mainly due to *Streptococcus Pneumoniae*, and herpes zoster infections. An increased propensity to develop lymphomas has been reported among later ADA-SCID presenters, usually related to infection with Epstein-Barr virus (EBV) or Cytomegalovirus (CMV) (Shovlin et al., 1993) (Kurlandsky et al., 1994).

### **1.8.3.2 Serum Immunoglobulins**

Total immunoglobulin levels may be only slightly depressed at birth due to the maternal contribution of IgG, whereas both IgM and IgA, which ordinarily do not cross the placental barrier, are often absent. However, once IgG levels decline as maternal antibodies are cleared (1 or 2 months of age), a pronounced hypogammaglobulinemia signals the absence of humoral immunity. Serum immunoglobulin levels are altered in later presenters, with IgG2 levels being generally highly reduced or absent. IgE levels are elevated and often associated to eczema and asthma. An inability to produce Abs against polysaccharide and pneumococcal Ags was frequently found in ADA-SCID patients with milder forms of the disease (Morgan et al., 1987) (Levy et al., 1988).

### **1.8.3.3 Autoimmunity**

A severe complication associated to milder forms of the disease is autoimmunity. Autoimmune manifestations include haemolytic anemia, type I diabetes, hypothyroidism and autoimmune thrombocytopenia (Notarangelo et al., 1992) (Ozsahin et al., 1997). Similar manifestation of haemolytic anemia has been reported in patients experienced long-term treatment with enzyme replacement therapy (Ratech et al., 1989), indicating that PEG-ADA is not sufficient to correct the immune dysregulation. Mechanisms of ADA-SCID-associated autoimmunity are not clear, although alterations of either central or peripheral tolerance could be responsible for these complications. Indeed, escaping or poor immune surveillance may be related to a different sensitivity of regulatory T cells to purine metabolites, as suggested by the finding that “suppressor” T cells are more sensitive than helper T cells, in drug-induced models of ADA-deficiency (Ratech et al., 1984).

### **1.8.3.4 Additional non-immunological abnormalities**

Since the ADA gene is expressed ubiquitously, the increased levels of purine metabolites cause also multisystemic pathologic changes in non-lymphoid tissues of

ADA-SCID patients (Ratech et al., 1989). Approximately 50% of the affected children exhibit detectable radiologically bony lesions, flared costochondrial junctions and “rachitic rosary”. Histological studies demonstrated the lack of organized cartilage columnar and trabecular formation with uninterrupted areas of calcified cartilage (Cederbaum et al., 1976). Anatomic malformations of the urinary tract and abnormalities in renal function, consequent to mesangial sclerosis, have been reported in some patients (Ratech et al., 1985). Neurological disturbances have been also documented and consist in mental retardation, spasticity, head lag, motor dysfunction, nystagmus and inability to focus (Rogers et al., 2001; Tanaka et al., 1996) (Albuquerque and Gaspar, 2004; Honig et al., 2007). Although the relationship of these disturbances to ADA-deficiency remains unclear, their improvement with the enzyme replacement therapy (Hirschhorn et al., 1980) has suggested that these abnormalities might reflect interaction of high concentration of Ado with adenosine A<sub>1</sub> receptors in nervous system.

### **1.9 ADA-SCID: Clinical management**

There are currently three different classes of therapeutic options available. They include: (1) bone marrow transplantation with histocompatible donors or with alternative donors; (2) enzyme replacement therapy; and (3) gene therapy.

#### **1.9.1 Bone Marrow transplantation**

Untreated severe ADA deficiency is usually fatal within the first years of life. BMT is generally the first choice therapeutic option for ADA-SCID patients for which an HLA-identical sibling donor is available (Antoine et al., 2003) (Stephan et al., 1993). This therapeutic manoeuvre displays a favorable positive outcome, with the 3-year survival post-BMT reaching over 90% (Antoine et al., 2003). This type of BMT is usually given without the requirement of prior conditioning, to reduce the risk of chemotherapy-associated toxicity, and generally results in a split chimerism, with T



lymphocytes of donor origin, while other lineages, including B cells, remaining host-derived (Antoine et al., 2003). This may lead to variable correction of B-cell deficiency and of the metabolic defect (Hirschhorn et al., 1981). Indeed, dAdo-based metabolites, even dramatically lower than in untreated patients, are still detectable higher than normal. Ado in the plasma persists at very high level apparently without any adverse effect. Haploidentical BMT with conditioning has resulted in elevated morbidity and mortality, with a 3-year survival of 29% (Antoine et al., 2003), in contrast to an overall better outcome for all forms of SCID (54%). The main reported causes of death were infections (56%), graft versus host disease (25%), and B-cell lymphoproliferative syndrome (5%). The occurrence of acute graft versus host disease led to poorer survival in related HLA-mismatched transplantation; no effect was apparent for chronic graft versus host disease. A significant improvement towards a better prevention of GVHD episodes in the non HLA-identical setting came from the first attempts to pursue BMT from T-cell depleted parental bone marrow early in life and without conditioning, to avoid toxicity of high dose chemotherapy (Buckley et al., 1999). The improved conditioning regimens and donor matches have opened new perspective for transplant from matched unrelated donors (MUD) or umbilical cord blood donors (UCB). However, there is currently limited information on the outcome of these transplants in ADA-SCID. Thus, in the absence of an HLA-identical sibling donor, BMT for ADA-SCID from an alternative donor remains a high-risk treatment.

### **1.9.2 Enzyme replacement therapy**

ADA enzyme replacement therapy was first evaluated in 1986 as non-curative, life-saving treatment for SCID lacking HLA matched bone marrow donors (Hershfield et al., 1987). A polyethylene-glycol-modified bovine ADA (PEG-ADA) was developed and commercialized based on the concept that reduction of ADA substrates concentration in plasma results in lowering of intracellular concentrations of metabolites

(Hershfield, 1995b). This chemical modification has allowed prolonging the circulating life of the enzyme, diminishing immunogenicity, and preventing degradation by proteases and binding by antibody. PEG-ADA is administered weekly or bi-weekly by intramuscular injection throughout life at an average dosage of 30-60 U/Kg. The drug acts in plasma to eliminate circulating dAdo and Ado, but does not cross the cell membrane. The kinetics and the extent of immune recovery vary between patients. In patients who respond to therapy, PEG-ADA results in immunological improvement (Weinberg et al., 1993) and decreases incidence of opportunistic infections (Hershfield, 1995a). However, the immune reconstitution is usually incomplete since most of the patients remain lymphopenic after the first year, *in vitro* lymphocytes responses to recall antigens have been inconsistent, and 50% continue to require IVIg replacement (Hershfield, 1998). Retrospective studies evaluating the long-term effectiveness of PEG-ADA treatment have indicated that the partial immune recovery may be related to decreased diversity of B lymphocyte and to the reduced thymic activity insufficient to compensate for the increased commitment to apoptosis of T lymphocytes in the periphery (Malacarne et al., 2005) (Chan et al., 2005). Enzymatic activity was found mostly in the plasma, with minimal amounts detected in tissues, as thymus and spleen. While circulating PEG-ADA may act as a metabolic sink detoxifying systemic levels of Ado and dAdo, it may not completely reduce intracellular levels in susceptible lymphoid progenitors or mature lymphocytes. In some cases (20%), patients treated with PEG-ADA show no or little improvement, with fatal course occurred due to causes related to the immune deficiency or with development of neutralizing antibody (Hershfield, 1998). In one fifth of the children who developed anti-ADA antibody (50-60%), the neutralization of ADA activity has required the increasing in dosage, administration of steroids or cessation of therapy (Hershfield, 1995a). Occurrence of autoimmune manifestation has also been reported (Hershfield, 1995a). More recently a

case of severe haemolytic anemia was observed in one patient, enrolled in PBL-gene therapy protocol in our institute, after reintroduction of PEG-ADA (Aiuti A., personal communication). The major drawback for the worldwide diffusion of enzyme replacement therapy consists in its high cost estimated € 200.000,00-400.000,00 per patient, annually.

### **1.9.3 Gene therapy**

Since the first pilot trials, gene therapy for ADA-SCID was supported by a strong rationale. The ADA gene is a housekeeping gene, widely expressed, for which a tightly regulated expression does not appear to be required. Low amount of correction and/or of engrafted HSC may result in successful therapy because as low as 10% of ADA activity can allow normal immune functions in healthy individuals (Hirschhorn, 1999) and wild type or gene corrected cells have a selective survival advantage over deficient cells in hematopoietic cell transplantation and preclinical gene therapy models (Ferrari et al., 1991) (Aiuti et al., 2003).

#### **1.9.3.1 Pilot gene therapy studies**

Pilot trials initiated in the 1990s were based on retroviral-mediated gene transduction but differed considerably with regards to the target cells, the retroviral construct, and the transduction procedure (reviewed in (Aiuti et al., 2003)). Patients eligible for gene therapy satisfied these inclusion criteria: absence of a histocompatible BMT sibling donor or poor immunological reconstitution upon replacement treatment. However, all patients continued to receive the PEG-ADA administration to avoid the risk of further impairing their incomplete immune functions, as the efficacy of gene therapy was under investigation.

#### **1.9.3.2 Gene therapy with peripheral blood T lymphocytes**

Three different trials reported the use of engineered peripheral blood T-lymphocytes (Blaese et al., 1995) (Bordignon et al., 1995) (Onodera et al., 1998). The

protocols employed repeated infusion of autologous T lymphocytes transduced with replication-deficient, recombinant retroviral vectors, derived from the Moloney Murine Leukemia virus (MMLV, see section 1.10) backbone, encoding ADA cDNA (Table II). The marker gene encoding neomycin resistance (neoR) was usually inserted in the vector to allow for identification and selection of transduced cells. The available follow up extends to over 13 years in the first treated children, with no adverse events or toxicity observed. The majority of the patients displayed substantial improved immune functions and transduced cells persisted many years after the infusions were discontinued, thus showing the safety and the therapeutic potential of PBL gene therapy. However, the real therapeutic impact of gene therapy was difficult to evaluate, because the concomitant administration of enzyme replacement therapy to all patients likely prevented the selective growth advantage of gene-corrected cells.

Investigators	Patients	Gene transfer protocol	Other treatments
Blaese et al. <sup>1,2</sup>	2	Transduction after stimulation with anti-CD3 mAb and IL-2	PEG-ADA
Bordignon et al. <sup>3</sup>	2	Transduction after stimulation with PHA and IL-2	PEG-ADA
Onodera et al. <sup>4</sup>	1	Same as 1	PEG-ADA
Aiuti et al. <sup>5</sup>	1	Same as 3	PEG-ADA then discontinued

**Table II. Gene therapy trials for ADA-SCID based on engineered peripheral blood lymphocytes.**

PEG-ADA, polyethylene glycol-adenosine deaminase; IL-2, interleukin-2.

1,2- (Blaese et al., 1995); (Muul et al., 2003)

3- (Bordignon et al., 1995)

4- (Onodera et al., 1998)

5- (Aiuti et al., 2002b)

### **1.9.3.3 Impact of PEG-ADA discontinuation on PBL gene therapy**

Despite the solid rationale for discontinuation of enzyme replacement therapy, as envisaged above, it was from an ethical point of view difficult to justify cessation of a potential beneficial treatment. Our group reported the discontinuation of PEG-ADA in a patient treated with PBL-gene therapy, which failed to sustain detectable immune responses and showed clinical features of immune imbalance during enzyme replacement treatment (Aiuti et al., 2002b). The protocol included a tapering down phase in which PEG-ADA dosage was gradually reduced whereas the infusion of transduced cells was intensified. In a non-detoxified environment, genetically corrected T-lymphocytes progressively replaced the untransduced counterpart leading to an increase in PBL ADA activity and, more importantly, to the correction of their functional defect (Aiuti et al., 2002b). However, the increase of purine metabolites in RBCs was associated with a decrease of B-cell number and immunoglobulin levels suggesting that the purine metabolic defect was only partially corrected (Aiuti et al., 2002b). This experience provided a strong rationale for a better approach allowing the correction of the immune defect and a sufficient systemic detoxification.

### **1.9.3.4 Gene therapy with hematopoietic stem cells**

HSCs were considered the optimal target cells to achieve the appropriate expression of the normal ADA gene in all haematopoietic cells. Because their long-lasting self-renewal and multilineage differentiation capacity, HSCs had the greatest potential to correct the immune defect and provide the highest level of systemic detoxification.

Eight patients were treated with genetically modified HSCs in the early trials (Table III) (Bordignon et al., 1995; Hoogerbrugge et al., 1996; Kohn et al., 1995). Cells were

collected from the BM or umbilical cord blood (UCB), enriched for HSCs, transduced and infused back into the patients without myeloablation.

Investigators	Patients	Gene transfer protocol	Other treatments
Bordignon et al. <sup>1</sup>	2	Infection of BM mononuclear cells with viral supernatant, no cytokines added	PEG-ADA
Kohn et al. <sup>2,3,4</sup>	3	Infection of UCB CD34 <sup>+</sup> cells with viral supernatant, in presence of cytokines (IL-3, SCF, IL-6)	PEG-ADA, discontinued briefly in one patient
Hoogerbrugge et al. <sup>5</sup>	3	Co-culture of BM CD34 <sup>+</sup> cells on irradiated producer with IL-3	PEG-ADA, in one of the patients started after 4 months
Aiuti et al. <sup>6</sup> (and unpublished data)	10	Infection of BM CD34 <sup>+</sup> cells with viral supernatant, in presence of retronectin and cytokines (IL-3, SCF, TPO, FLT3 ligand)	No PEG-ADA; low intensity conditioning with busulfan
Candotti et al. <sup>7</sup>	4	Infection of BM CD34 <sup>+</sup> cells with viral supernatant, in presence of retronectin and cytokines ( SCF, TPO, FLT3 ligand)	PEG-ADA; no conditioning
Gaspar et al. <sup>8</sup>	2	Infection of BM CD34 <sup>+</sup> cells with viral supernatant, in presence of retronectin and cytokines ( IL-3, SCF, TPO, FLT3 ligand)	No PEG-ADA; mild intensity conditioning with melphalan

**Table III. Gene therapy trials for ADA-SCID based on engineered haematopoietic stem cells.**

SCF, stem cell factor; TPO, thrombopoietin; IL-3, interleukin-3; IL-6, interleukin-6; PEG-ADA, polyethylene glycol-adenosine deaminase.

1- (Bordignon et al., 1995)

2,3,4- (Kohn et al., 1995); (Kohn et al., 1998); (Schmidt et al., 2003)

5- (Hoogerbrugge et al., 1996)

6- (Aiuti et al., 2002a)

7- (Candotti et al., 2003)

8- (Gaspar et al., 2006)

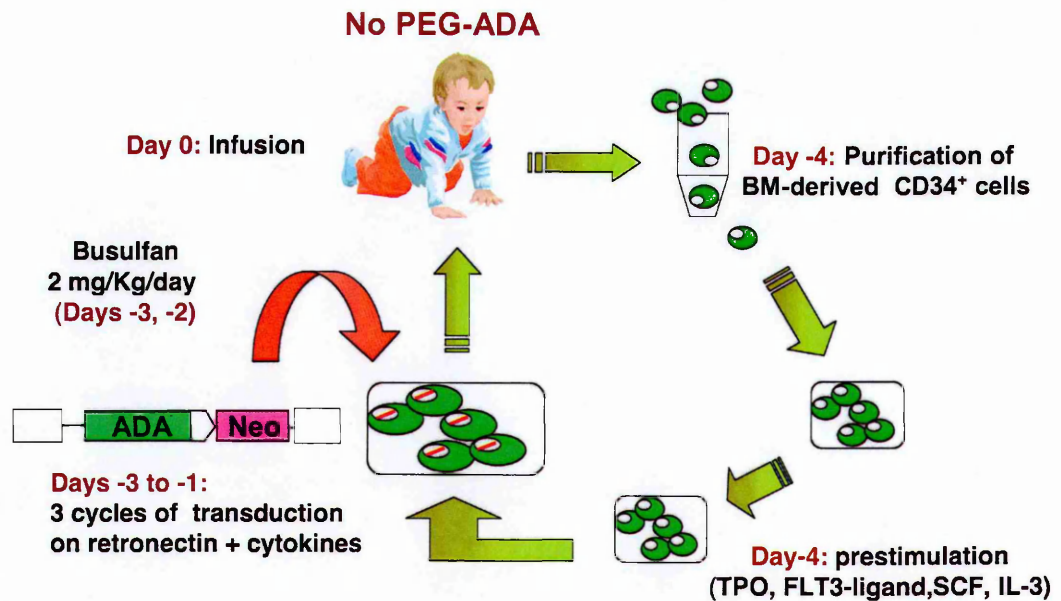
Patients enrolled in the HS Raffaele trial received multiple infusions of BM-enriched progenitors cells and PBLs, transduced with similar vectors but carrying different molecular markers (Bordignon et al., 1993). This study reported a progressive replacement of circulating T cells derived from transduced PBLs with BM-derived T

cells and, after two years of treatment, the long-term survival of BM-derived transduced cells was demonstrated in T-, B-lymphocytes, granulocytes and BM cell subsets. In the three patients that received genetically-corrected UCB CD34<sup>+</sup> cells, the four years follow up still indicated the presence and expression of the ADA gene in the BM and PB (Kohn et al., 1995). However, no conditioning was administered to the patients and the level of engraftment of transduced cells was insufficient for achieving ADA expression at therapeutic levels (Kohn et al., 1998). In addition, the efficacy of gene therapy was difficult to evaluate because all patients continued to receive enzyme replacement therapy. Overall these results represented a proof of principle of the feasibility and safety of infusion of engineered stem/progenitor cells, paving the way to new clinical trials based on improved design and protocols.

#### **1.9.3.5 HCS gene therapy combined with non-myeloablative conditioning**

A novel clinical study based on improved gene transfer protocol combined with a strong selective advantage for the engraftment and the expansion of HSCs and their lymphoid progeny was carried out at the HSR-TIGET (Aiuti et al., 2002a) starting from September 2000. Transduction protocol of HSCs was optimized using retroviral supernatant produced under conditions adapted for human CD34<sup>+</sup> cells and a culture medium containing a cocktail of cytokines (Illustration VIII) (Dando et al., 2001) (Ficara et al., 2004). In order to provide an initial advantage for adequate HSCs engraftment, low-intensity conditioning was introduced before the infusion with autologous gene-corrected cells (Aiuti et al., 2002a). The rationale for the use of non-myeloablative regimen originated from gene marking studies in animal models and the recent experience with BMTs for haematological disorders (Huhn et al., 1999) (Rosenzweig et al., 1999) (Slavin, 2002). Busulfan was chosen as a single chemotherapeutic agent because of its large use in BMT for pediatric patients, including patients with SCID (Bolinger et al., 2000). The dosage utilized (2mg/Kg for two

consecutive days) represented about 25% of the typical dosage used in full myeloablative regimens.



*Aiuti et al. Science, 2002*

**Illustration XIV. Gene transfer protocol into autologous BM-CD34<sup>+</sup> cells of ADA-SCID patients.** CD34<sup>+</sup> cells were harvested and purified from BM and prestimulated for 24h in the presence of cytokines. Subsequently, cells were transferred to retronectin-coated bags previously bated with retroviral particles and further transduced twice, for a total culture period of 4 days. Before reinfusion of the gene corrected cells, the patients received busulfan from day -3 to -2, at a total dose of 4 mg/kg.

Two patients, who did not have access to PEG-ADA, were enrolled in the initial phase of the trial. This condition enabled to fully exploit the selective advantage for gene-corrected cells and to evaluate the clinical efficacy of gene therapy alone. Using this protocol, gene therapy resulted in sustained engraftment of engineered HSC which differentiated in multiple lineages, increased lymphocyte counts, improved cellular and humoral responses, including antigen-specific responses, and in a substantial reduction in the toxic metabolites (Aiuti et al., 2002a). These results represented the first demonstration of the clinical efficacy of HSC gene therapy alone for ADA-SCID and



have been now extended to additional eight patients treated with the same protocol, of which six have been followed up for more than one year. All patients are healthy and show partial to full immune reconstitution, with improved systemic detoxification (Aiuti et al., *manuscript in preparation*). More recently, another group reported a successful HSC gene therapy protocol combined with mild chemotherapy regimen in one patient who showed poor response to PEG-ADA (Gaspar et al., 2006). The human ADA cDNA expression was driven, in this case, by the SFFV LTR of a gammaretroviral vector, which also contained a mutated WPRE. Two years after the procedure, immunological and metabolic correction has been maintained, with reconstitution of cellular immunity, reinitiation of thymopoiesis and systemic detoxification, in absence of any side effect.

#### **1.9.4 Open issues in gene therapy with HSC**

A key issue determining the final outcome of HSC gene therapy remains the degree of chimerism. Despite the overall improvement of both the immune and metabolic defect of ADA-SCID children enrolled in the clinical trial, one patient, displaying limited engraftment of gene-corrected stem/progenitor cells, achieved partial immune recovery and systemic detoxification (Aiuti et al., 2002a). Several explanations may be accounted for the reduced level of engraftment: 1) the significant lower number of transduced CD34<sup>+</sup> cells infused, 2) the older age, which can be crucial factor for HSC engraftment, as shown for BMT transplantation in SCID (Buckley et al., 1999), 3) the reduced degree of BM ablation, since the patient received oral Busulfan. Therefore, establishing the correct balance between the amounts of transduced HSC that need to be infused to get long-term engraftment and the safety and low toxicity of the current conditioning regimen appears critical in the determining the definitive successful outcome of gene therapy. Moreover, it is critical to reach an adequate size of the gene corrected compartment, to allow sufficient degree of systemic detoxification and immune recovery. Thus, understanding which is the minimal level of ADA enzyme

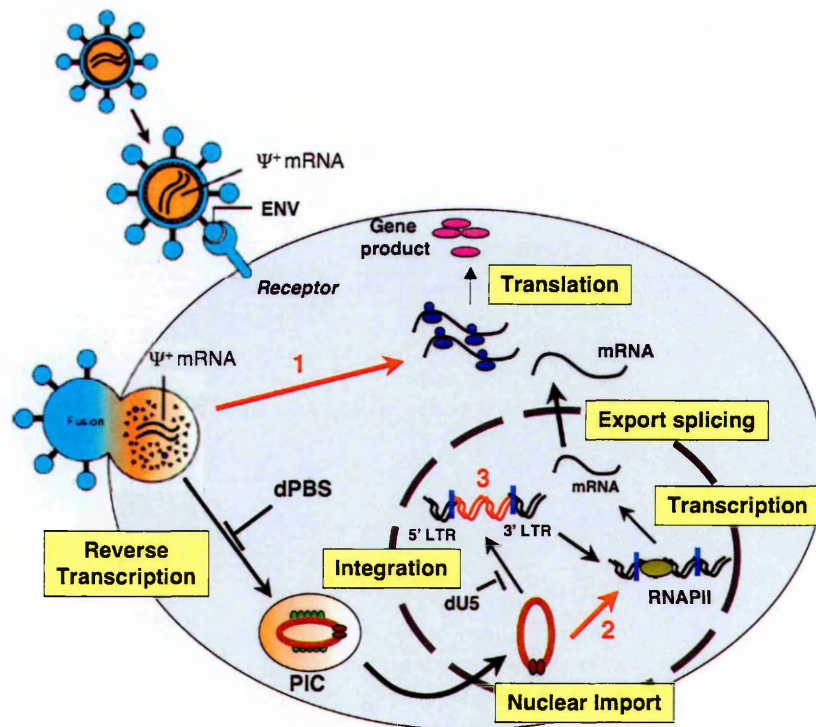
activity sufficient to ensure metabolic and immune reconstitution becomes important and it would also be useful for future improvement of GT protocol. In this regard, it has been reported that ADA activity in leukocytes of children with partial ADA deficiency, which is due to mutations leading to absence of ADA in erythrocytes but residual activity in other cell types and usually associated with normal immune function (Jenkins, 1973), ranges between 2-10% of normal (Jenkins et al., 1976). It is conceivable that these levels are close to the threshold required for normal immune function. Besides the safety record of all ADA-SCID gene therapy protocols, the adverse events occurred in one of the SCID-X1 GT trials have raised general concerns on the potential risk of insertional mutagenesis caused by retroviral vector (RV) integration, and the long-term safety of gene therapy is now being a crucial open issue. The potential and the actual side effects of RV gene transfer will be discussed in detail in sections 1.11 and 1.12.

### **1.10 Retroviral vectors: Murine leukaemia virus**

Among the vectors currently available for gene therapy, retroviral vectors (RV) are the most widely used. Most of them, as the one used in our HSC gene therapy trial, were derived from the retrovirus MMLV (Moloney murine leukemia virus) (reviewed in (Verma and Weitzman, 2005)). The genome of MMLV is constituted by a single strand RNA, which, after infection of the host cell, is transcribed into a double strand DNA molecule, by the viral enzyme reverse-transcriptase (pol) (Illustration XV). Transport of the viral DNA to the nucleus requires nuclear membrane breakdown occurring during mitosis (Roe et al., 1993). Therefore, MMLV and MMLV-derived retroviral vectors are able to infect only dividing cells. The viral genome, flanked at either ends by two identical LTRs, is constituted by three main genes: gag, pol and env, which encodes respectively for structural proteins (gag), reverse transcriptase and integrase (pol), and

proteins of the viral envelope (env). The peculiarity of retrovirus, which make them suitable and safe vectors for gene therapy, is that the only elements required for viral replication and packaging in cis are represented by the 5' and 3' long terminal repeats (LTR), which contain promoter, poly-adenylation sequence and the packaging site ( $\psi$ ). All the other proteins can be supplied in trans. Therefore, all the genes encoding for proteins necessary to produce infectious particles can be removed and replaced with a cDNA encoding for the gene of interest. Transcription of this cDNA can be driven either by viral LTR or by an internal promoter. Since it is often necessary to express two genes within the same retroviral vector (usually one is the gene of interest and the other is a selectable marker gene), different strategies have been exploited to this purpose.

One possibility is to use the viral LTR to drive the expression of one gene and an internal exogenous promoter to drive the expression of the second gene. However, the presence of two promoters, in the context of the same vector, reduces cDNA expression. Therefore, alternative strategies have been developed, such as the use of a polycistronic message with the addition of an internal ribosome entry site (IRES) (Zitvogel et al., 1994). To produce RV, packaging cell lines have been developed. GP+Am12 (Freas-Lutz et al., 1994) is a third-generation packaging cell lines, generated by stably transfecting NIH 3T3 cell line with two different plasmids, encoding for gag-pol and env proteins, respectively. Vector production by the packaging cell line is achieved by stable or transient transfection with the vector plasmid, derived from the vector backbone and containing the  $\psi$  sequence and the gene(s) of interest. Safety of this system is guaranteed by the fact that three independent events of recombination would be necessary to produce a replication competent virus.



**Illustration XV. Delivery of nucleic acids by retroviral particles.** Following receptor-mediated uptake (by fusion or via endosomes, depending on the envelope protein), retroviral particles can deliver three forms of genetic information: (1) if reverse transcription does not occur, the mRNA may be subject to immediate translation; (2) if integration is blocked, episomal circles can be generated that may persist in non-dividing cells; (3) if all steps of the retroviral transduction process are completed, a double-stranded DNA integrates in cellular chromosomes. DPBS, deletion/mutation of the PBS, Datt, deletion/mutation of the att sites; RNAPII, RNA polymerase II.

GP+Am12 is an amphotropic packaging cell lines, since it express an envelope, which allows the infections of both murine and human cells. Soon after, another murine packaging cell was made, the PG13 cell line, which differed from the GP based packaging cells for the use of a different envelope. The envelope was derived from Gibbon Ape Leukaemia Virus (GALV) retrovirus, which can efficiently be pseudotyped with the MLV Gag-pol (Miller et al., 1991) and targets a receptor very similar but not identical to the amphotropic receptor (Miller and Miller, 1994).

## **1.11 Side effects evaluation of RV-mediated gene transfer**

### **1.11.1 Risks related to vector production**

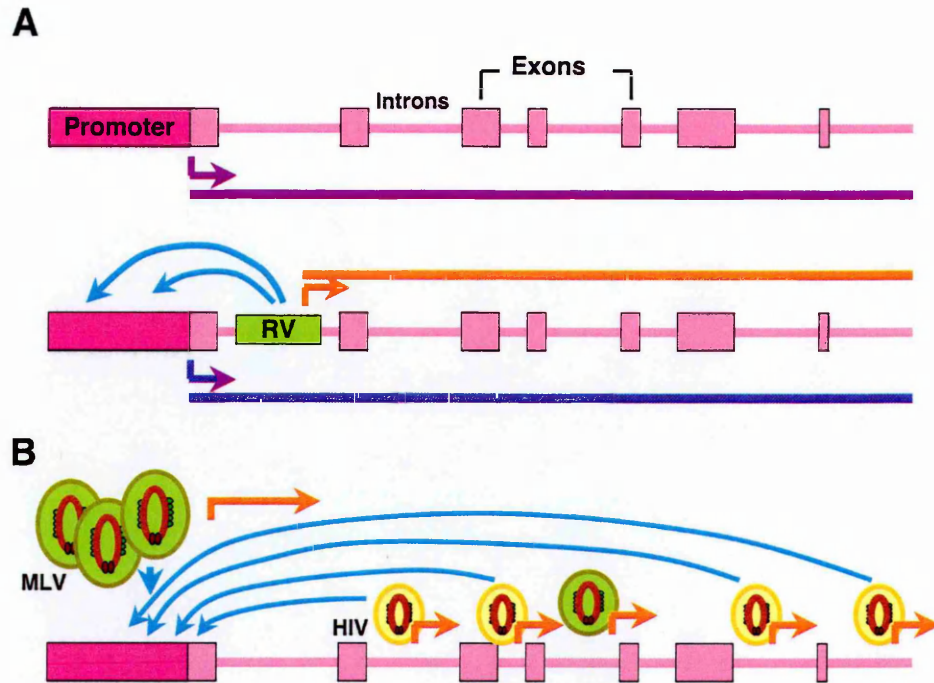
As new biological intended to be used in the clinic, RV has to comply with a complete set of requirements regarding their mode of preparation and characterization. The possibility of generating replication-competent retrovirus (RCR) during vector preparation or use of gene transfer vectors is a major concern associated with any virus-derived system. Recombination events that result in the formation of RCR occur between the recombinant vector genome, which carries all sequences needed in *cis* for packaging, reverse transcription and integration, and the sequence providing the viral proteins in trans, within the packaging system. This can happen at the DNA level between: (i) co-transfected plasmid; (ii) a transfected plasmid and a chromosomal sequence bearing homologous sequences; (iii) the proviral vector DNA generated after target cell transduction and chromosomal sequence. Much more frequently in RV system, recombination occurs at the RNA levels.

### **1.11.2 Risks related to transgene insertion**

Due to the fact that during replication, viral RNA genome was reverse transcribed into a DNA copy that becomes integrated into the host cell genome, RV may theoretically induce cell transformation by a phenomenon known as insertional mutagenesis. Historically, this risk has been generally evaluated as very low, basing on the probability of insertion in a given site of the host cell genome. Unfortunately, clinical adverse events, linked to vector integration leading to *LMO2* proto-oncogene transactivation, occurred in otherwise successful HSC-based gene therapy trial of SCID-X1 (Hacein-Bey-Abina et al., 2003) (see section 1.12). These events have raised serious concerns on the use of RV in clinical application and the safety of this treatment has become a primary consideration.

#### **1.11.2.1 Patterns of integration of RV vectors**

The readily accessible human genome sequence and the development of robust PCR-based technologies prompted to the diffusion of mapping studies of RV integration sites (RIS) with regard to their exact chromosomal location, relation to neighboring sequences and potential interference with coding and regulatory regions. A comparative analysis of cell lines, non-human primate and human hematopoietic progenitors cells transduced with HIV-1 or MLV based vectors showed that each vector type produces a unique pattern of RIS distribution in the human genome. MLV integrants were found preferentially surrounding the transcription start sites (TSS) and regulatory regions (CpG islands) of active genes, while HIV preferentially targeted intragenic sites with a distribution along the entire length of the active gene (Schroder et al., 2002; Wu et al., 2003) (Mitchell et al., 2004) (Hematti et al., 2004) (Laufs et al., 2004). Moreover, studies with gene trap vectors performed in primary hematopoietic cells confirmed the stronger bias of MLV vectors to insert in promoter-proximal windows, as compared with similarly designed HIV-derived vectors (De Palma et al., 2005). Therefore, MLV vector integrations near 5' ends of genes may be more likely to disrupt transcriptional control and result in deregulated expression of potentially oncogenic gene products, while the main risk of LV could be the abrogated production of the normal gene (Illustration XVI). A striking finding in this large-scale integration survey was the evidence for the formation of integration hotspots (Mitchell et al., 2004; Schroder et al., 2002) (Calmels et al., 2005) (Kustikova et al., 2003). Recurrent integration in specific gene loci has provided evidence that the insertion may confer a non-random growth or survival advantage to the affected cell clones (Calmels et al., 2005; Kustikova et al., 2003). This aspect will be discussed in section 1.11.2.3.



**Illustration XVI. Activation of cellular genes by insertional mutagenesis.** (A) A prototypic gene, for instance an oncogene, is shown with its promoter, exons, and introns. The primary transcript originating from the promoter is represented as a purple line. Integrations of a retrovirus or a retroviral vector near the promoter can stimulate the latter via enhancer elements located in the virus LTR or internal promoter (blue arrows), resulting in the increased production of the physiological primary transcript (dark blue line). Aberrant transcripts can also originate from promoters contained in the vector itself (orange line). (B) MLV vectors (green) integrating in and around the promoter have a high chance of stimulating cellular gene expression by either enhancer (blue arrows) or promoter (orange arrows) effects. The transcript emanating from vector-contained promoters will be full-length or nearly full-length. As they integrate farther away along the transcribed region of the gene, HIV vectors (yellow) may have a lower chance of stimulating the cellular promoter via enhancer effects, and can only give rise to significantly truncated version of the normal cellular transcript.

#### 1.11.2.2 Factors influencing integration site selection

The integration reaction requires specific sequences at the double strand ends of the viral cDNA, which bind the viral-encoded integrase and other proteins to form preintegration complexes (PICs; (Schroder et al., 2002)). Studies using integration *in vitro* have begun to clarify factors influencing integration site selection in simplified models. DNA-binding proteins bound to target DNA create “hot spot” for integrations. The mechanism is still unclear, but DNA distortion around nucleosomes may favor the access of the viral integration complexes to receptive DNA (Bushman, 1994).

Integration may be promoted by increased chromatin accessibility in transcribed regions through favorable interactions between PICs and locally bound transcription factors or chromosomal proteins. In support of this idea, it has been established that retroviral integrase enzymes fused to sequence-specific DNA-binding domains can direct integration preferentially to local regions when tethered at specific DNA sites (Bushman, 1994). Thus, the ultimate pattern of vector integration may reflect both the integration preferences of the vector system used and the nature of the regulatory elements included within the vector. The analysis of chromosomal regions favored for integration confirmed a role for locally bound proteins. Statistical test have indicated that favourable regions were typically short (100-250 Kb) and for MLV these landed within or near CpG islands. CpG islands are thought to be regulatory regions that bind distinctive sets of transcription factors. Therefore, a distinctive set of sequence-specific DNA-binding proteins bound at or near CpG islands favour MLV integration, while proteins bound in active transcription units are favourable for HIV insertion (Mitchell et al., 2004). A further possibility is that the intranuclear environment of active genes favors integration. However, whether transcription promotes integration directly or by indirect mechanisms is currently unclear.

#### **1.11.2.3 Hot spots for retroviral integration**

Analysis of MLV integrations pattern in natural or experimentally induced leukaemia/lymphomas have shown the existence of insertion sites recurrently associated with a malignant phenotype. These “common insertion site” (CIS) include proto-oncogenes or other genes associated with cell growth and proliferation, the activation or deregulation of which has a causal relationship with the establishment and/or progression of neoplasia (Wu et al., 2006). Some of these sites, such as the EVI1-MDS1 locus, have been identified at relatively high frequency also in the non-malignant progeny of transduced hematopoietic cells in mice (Kustikova et al., 2005) and non-



human primates (Calmels et al., 2005), indicating that insertion into certain genes may cause clonal amplification of transduced progenitors *in vivo*. More recently, insertions into EVI1-MDS1 locus and into the related gene, PRDM16, have been found in the dominant clones with myeloid expansion in the two patients of the GT trial of CGD (see section 1.12), suggesting that these genes can influence regulation of normal long-term hematopoiesis in humans. The lack of progression towards monoclonality or leukemia, observed in these studies, would indicate that these events likely depends on the occurrence of additional mutations or on cooperating effects of the transgene. Low incidence of CIS insertion has been reported for human gene-modified T lymphocytes (Recchia et al., 2006).

#### **1.11.2.4 Impact of integration on expression of nearby genes**

Detailed information is beginning to accumulate about the impact of specific integration events on the expression of nearby genes, a phenomenon known as transcriptional perturbation. Dominant clones that contributed to hematopoiesis in secondary transplant recipients were consistently found to have increased expression of a gene within the region of the integrated RV genome that could be implicated in potentially causing proliferation or survival advantage (Kustikova et al., 2003). A large scale-mapping of RIS in modified T lymphocytes from leukemic patients after allogeneic stem cell transplantation has shown that RV vectors integrated preferentially around TSS and in genes expressed during transduction (Recchia et al., 2006). Some integrations, landed within 25 Kb upstream from the TSS or more than 100 Kb downstream, can deregulate gene expression independently from their transcriptional orientation, albeit without consequences on the biology and function of transplanted cells (Recchia et al., 2006). Another continuing study has examined the impact of LV vectors integrated into primitive hematopoietic cells (Hargrove P. W. , 2004). Despite the occurrence of rare cases of transcriptional perturbation in genes some distance (as

far as 300Kb) from the integration site, the majority of integrated vector genomes had no discernible impact on expression of nearby genes even when the integration had occurred in a gene-rich area.

#### **1.11.2.5 Silencing**

Vector integration into, or next to heterochromatin may induce, through specific retroviral enhancer elements, inhibition of transcription (position-dependent silencing) or may result in varying expression in progeny cells (position-effect variegation) (Brenner et al., 2003). Indeed, it is commonly agreed that gene silencing tends to occur in undifferentiated cells that go through a long differentiation process. CpG methylation is strongly correlated with gene silencing. Regions rich in nonmethylated CpG are found in many functional promoter elements. Integration of foreign DNA sequences into the genome itself triggers methylation of CpG dinucleotides and DNA methylation inhibits transcriptional activity. A link between DNA methylation and chromatin structure has recently been identified. By binding to methylated viral sequences, host proteins mediate the recruitment of histone deacetylases (HDAC) to the transduced gene locus. HDAC deacetylate specific histones and lead to chromatin condensation and transcriptional silencing (Wolffe and Matzke, 1999).

#### **1.11.3 Risks related to transgene expression**

Using available vectors and HSCs as targets, somatic gene transfer typically results in ectopic and nonregulated expression of the transgene, both with respect to the cell type affected and the level of expression achieved. Moreover, the repertoire of vector integration sites may also modulate different aspects of the transgene expression, including duration, level and differentiation dependence. The ectopic and deregulated expression of the transgene may cause, in some instances, dose-dependent toxicity (Schiedlmeier et al., 2003) or even severe side effects (Zhou et al., 2001). Toxicity related to transgene expression may most frequently manifest in a competitive

disadvantage, leading to extinction of the affected cell (clone). However, transgene interference with cellular behavior may eventually results in new disease manifestation. Therefore, it can be predicted that any transgene product has a specific therapeutic window, defined by the balance between desired function and unwanted effects. The exact definition of this window, which would be crucial for the evaluation of gene transfer therapeutic potential, may depend on the ability to maintain a regulated expression, thus highlighting the importance of developing vectors for spatially and temporally controlled expression of the transgenes.

#### **1.11.4 Risks related to conditioning**

Following genetic modification of HSCs *in vitro*, their engraftment and contribution to hematopoiesis *in vivo* are dependent upon the strategies used for conditioning or selective amplification. Irradiation or cytotoxic agents, which provide a moderate to severe (myeloablative) lymphohematotoxicity, can however be complicated by severe long-term toxicity. Nonmyeloablative regimens with sublethal toxicity have been investigated (Sandmaier et al., 2000) and show great promise for HSC-mediated gene therapy (Aiuti et al., 2002a). However, it remains unclear whether chimerism will be maintained in a stable manner, long term, when nonmyeloablative protocols are performed in autologous clinical setting. Indeed, key factors such as incomplete tolerance and absence of selective advantage for engineered cells may determine the final outcome. A selective survival advantage for gene modified HSCs can be promoted upon transfer and expression of selectable marker genes (Mavilio et al., 1994) (Li et al., 2002). For most of these markers, drugs are required to trigger their function (Bonini et al., 1997). Therefore, side effects related to their use represent an important aspect in the preclinical and clinical evaluation. Indeed, while some of these agents have a well-documented toxicity profile in humans (Sorrentino, 2002), others potentially less toxic may provide incomplete, lineage restricted or unstable expansion (Matsuda et al., 1999).

Clonal amplification of transgenic cells is another important variable. Although gene-marking studies have indicated that the size of grafted stem cells implies a modest expansion pressure and a high likelihood for polyclonal reconstitution (Schmidt et al., 2002), single clones of transduced HSCs may achieve normal multilineage hematopoiesis (Pawliuk et al., 1997). However, the minimal number of HSCs that stably support primate hematopoiesis remains to be defined.

#### **1.11.5 Risks related to immune surveillance**

A further category of side effects is related to innate or acquired immunity against vector components or immune surveillance of engineered cells. In principle, a transient exposure to antigens may derive from remnants of vector particles or culture media components on infused cells (Balague et al., 2000). This risk appears relatively low with the conventional retroviral transduction protocols, but the probability of viral contamination may increase with the use lentiviral vectors, for which the time of cell culturing after the final round of vector exposure may be shortened (Scherr et al., 2002). Accordingly, repetitive infusion of engineered cells may be complicated either by sensitization to vector- or culture-related antigens, or transgene expression, potentially resulting in clearance of transgenic cells (Tuschong et al., 2002) or even severe acute adverse reactions. Immune responses against transgene may develop with some latency. This concern is of particular relevance when introducing artificial or xenogenic sequences (as in the use of some selectable marker genes). Indeed, although BMT may promote tolerance to multiple or individual antigens (Heim et al., 2000) (Kang et al., 2001), this does not necessarily occur following nonmyeloablative conditioning regimens. Immune-mediated rejection of transgenic cells expressing the xenogenic marker enhanced green fluorescent protein has been documented in a study with non human primates (Rosenzweig et al., 2001), suggesting that tolerance induction should be a key issue for future gene therapy applications.

### 1.12 Genotoxic effects of RV gene transfer in GT clinical trials: the paradigm of SCID-X1 and Chronic Granulomatous Disease

Recently, the potential genotoxic effect of RV has dramatically come to the attention, due to the occurrence of adverse events in one ongoing RV-based clinical trial for SCID-X1. The genetic defect originates from the lack of the  $\gamma$  subunit of the IL-2 receptor, also called the common  $\gamma$  chain (Kovanen and Leonard, 2004). Gene therapy trials were based on the use of MMLV-derived retroviral vectors. Autologous BM-derived CD34<sup>+</sup> cells were transduced *ex vivo* and then re-infused into the SCID patients in absence of any preparative conditioning (Cavazzana-Calvo et al., 2000) (Gaspar et al., 2004). In the French trial, the effectiveness of the treatment was demonstrated in 9 out of 11 patients enrolled with sustained transgene expression and immunological reconstitution (Cavazzana-Calvo et al., 2005). However, three patients developed leukaemia, approximately 2.5 years after gene therapy. Genetic analysis of the leukemic clones showed that in the two first cases the retroviral-vector had integrated within or upstream of the *LMO2* locus causing an insertional activation of the proto-oncogene transcription mediated by retroviral LTR. The third case also seems to be associated with this event, among additional proto-oncogene insertions (Hacein-Bey-Abina et al., 2003) (<http://afssaps.sante.fr/ang/indang.htm>). *LMO2* is normally expressed in haematopoietic progenitors and its locus is involved in chromosomal translocation in cases of acute T-cell leukaemia (T-ALL) (Rabbitts et al., 1999). Although vector integration close to *LMO2* gene was found to reside within FRA11E, a common fragile site (Bester et al., 2006) (Hacein-Bey-Abina et al., 2003) and chromosomal aberrations were detected in the expanded clones of both patients, the mature phenotype of such clones cannot be associated with T-ALL (Hacein-Bey-Abina et al., 2003). No overexpression of  $\gamma c$  gene or constitutive activation of downstream transducing molecules was observed in either the normal corrected or the leukemic T cells from

patients (Hacein-Bey-Abina et al., 2003). Furthermore, gain-of-function mutations of the  $\gamma c$  receptor were excluded by sequencing the integrated provirus (Hacein-Bey-Abina et al., 2003). Beside these findings, evidence has emerged recently supporting the hypothesis that RV-driven  $\gamma c$  transgene expression may synergistically cooperate to the oncogenic transformation, possibly influencing T cell differentiation (Dave et al., 2004) and even act as an oncogene itself in murine models of leukemogenesis (Woods et al., 2006). However, this interpretation have been challenged other studies indicating the inability of overexpressed *IL2R $\gamma c$*  to affect normal T cell development (Pike-Overzet et al., 2006; Pike-Overzet et al., 2007) and increase tumor incidence in different mouse models (Thrasher et al., 2006). Thus, the potential interaction, if any, between *LMO2* overexpression and RV-mediated  $\gamma c$  gene expression remains uncertain. No vector integration in *LMO2* oncogene was detected in the English trial (A. Thrasher, oral presentation, 3<sup>th</sup> Stem Cell and Genotoxicity Retreat, 2007, Orlando). Therefore, additional factors may be more related to the gene therapy protocol, such as *ex vivo* culture conditions, vector pseudotype or the kinetic of lymphoid reconstitution. The results of the HSC gene therapy trial for CGD, gained by Ott and colleagues, throw different light on the influence of transcriptional perturbation on gene therapy outcome. CGD is a rare inherited immunodeficiency caused by a functional defect in the microbial killing activity of phagocytes (Segal, 1996). Almost 70% of CGD patients carry defects in the X-linked gene encoding for gp91phox (X-CGD) (Winkelstein et al., 2000), essential subunits of the phagocytic NADPH oxidase complex. The clinical protocol was based on *ex vivo* retroviral gene transfer of gp91phox cDNA into (G-CSF)-mobilized PB CD34<sup>+</sup> cells combined with non-myeloablative conditioning (L-Bu) (Ott et al., 2006a). Results of the trial have indicated the restored enzyme activity in transduced phagocytes, which was paralleled by the resolution of natural occurring bacterial and fungal infections in the three patients enrolled (Ott et al., 2006a) (Ott et al.,

2006b). The high level of correction achieved in phagocytes resulted from an unexpected *in vivo* expansion of gene corrected myeloid cells, due to activating common integration sites in the zinc finger transcription factor homologs *MDS1/EVII*, *PRDM16* or *SETBP1* (Ott et al., 2006a). Among the three clusters of integrations, the relative contribution of each clone changed over time with dominant clones retaining the major stable contribution. Unfortunately, a progressive decrease in the number of gene corrected cells and in the functional activity of the predominant clone was observed more than two years after GT in Pt1, leading to his fatal course due to severe bacterial sepsis (Ott et al., 2006b). Although the molecular causes are still to be determined, vector silencing consequent to methylation of the R and U5 LTR regions seems to be implicated (M. Grez, oral presentation, 3<sup>th</sup> Stem Cell and Genotoxicity Retreat, 2007, Orlando). Importantly, no evidence of malignant transformation was found in peripheral blood or bone marrow aspirates from this patient, as demonstrated also by growth factor-dependent proliferation of transduced cells.

Several factors, primarily related to the gene transfer protocol, could be accounted to explain the clonal expansion/selection observed in CGD trial. The use of a vector with SFFV long terminal repeat sequences, which contains potent enhancer elements for gene expression in hematopoietic stem and myeloid progenitors cells, may have favored the activation of specific genes and led to the observed clonal over-representation. Integrations in *MDS/EVII* and *PRDM16* genes were also described in other gene therapy studies (see section 1.11), suggesting that these genes can influence regulation of normal long-term hematopoiesis in humans. Although longer follow up is required for safety considerations, the experience of CGD trial lead to consider additional implications in the balance between clinical benefit and the potential risk associated to the type of treatment. Indeed, the therapeutic benefit was achieved in treated CGD patients through the insertional side effects of gene therapy approach, which provided

selective growth advantage for affected cells.

### **1.13 Future perspective for safety improvement**

Third generation lentiviral vectors are constructed in a way to address the biosafety requirements, which implies the removal of all the proteins involved in AIDS pathogenesis, while all the other structural and enzymatic proteins are provided in trans in three different plasmids. These vectors were shown to efficiently transduce human BM-derived repopulating CD34<sup>+</sup> cells after short ex vivo manipulation (Guenechea et al., 2000) and to provide high level and stable transgene expression, making them a suitable tool for gene therapy trials in haematological diseases (Mortellaro et al., 2006).

The use of cellular instead of strong viral promoters would allow regulated and tissue-specific expression of the therapeutic gene, avoiding ectopic expression and reducing the potential toxicity of the treatment. As for RV, LV carries the risk of insertional mutagenesis. In spite of this, LV are likely to become the vectors of choice for the future gene therapy clinical trial, as they accomplish two important safety requirements: the usage of transcriptional regulatory elements and low genotoxicity due to the possible influence of the vector on the transcription of neighbouring genes (Montini et al., 2006). However, to demonstrate that LV would be less prone than their oncoretroviral counterparts to cause tumors by insertional mutagenesis, more extensive and long-term studies in relevant animal models need to be pursued.

The construction of vectors with the so-called self-inactivating (SIN) LTRs, which rely on a single internal promoter to drive transcription of the transgene (Miyoshi et al., 1998), would significantly reduce the risk of aberrant expression of adjacent genes. This was achieved by creating a deletion in the U3 region of the 3' LTR. During reverse transcription in the target cells, this deletion is transferred to the 5' LTR of the proviral DNA. If the deletion is efficient to abolish the transcriptional activity of the LTR promoter, the transcription of the full-length vector RNA would be eliminated in



transduced cells. The possibility of insertional activation of adjacent cellular oncogenes would also be reduced. Furthermore, as there would be no complete U3 sequence in the producer system, recombination to generate a wild type U3 would not be possible. In addition to improved biosafety, the use of SIN vectors has two added advantages: elimination of transcriptional interference by LTR promoter, and the possibility to create tissue-specific and inducible vectors, which would be difficult in the presence of non-specific transcription from the LTR promoter (Zufferey et al., 1998).

Insulators are DNA elements with enhancer-blocking activity that can also be engineered into therapeutic vectors to inhibit transactivation of adjacent genes in the genomic DNA (West et al., 2002). In oncoretroviral vectors, insulators have been shown to attenuate position effects (Rivella et al., 2000), but further investigations in appropriate animal models would be required for efficacy evaluation. A different approach to tackle the risk of insertional oncogenesis would be to incorporate into vector a safety function that could be activated in the case of tumor formation, as proposed for the elimination of alloreactive T lymphocytes (Bonini et al., 1997). The functional reliability, the transcriptional regulation and the immunogenicity of existing suicide systems remains to be established.

### 1.14 SPECIFIC AIMS

ADA-deficient SCID is a complex genetic disease with the major involvement of lymphoid compartment in the disease pathogenesis. Different mechanisms whereby ADA deficiency may affect the development and function of T lymphocytes have been suggested, including ADA substrates accumulation and aberrant adenosine receptor signaling. However, the precise mechanisms of this toxicity are not yet defined in humans, as no in depth studies have been performed in ADA-SCID patients.

ADA-deficiency can be successfully treated with HSC-based gene therapy. Results of the clinical trial established at HSR-TIGET have shown the correction of the immunological and metabolic defect in the patients enrolled, with full clinical benefits in the absence of side effects (Aiuti et al., 2002a). However, despite the excellent safety record of ADA-SCID trial, the long-term safety of retroviral-mediated therapeutic gene transfer has been recently questioned based on the risk of insertional mutagenesis, which has emerged as a serious drawback in the context of GT for SCID-X1 (Hacein-Bey-Abina et al., 2003).

The main aims of my PhD project were to study the molecular basis of ADA-SCID pathogenesis and to evaluate the efficacy and safety of HSC gene therapy as a curative treatment for the disease.

Untransformed polyclonal T-cell lines established from ADA-SCID patients before and after gene therapy have provided a unique model to investigate i) the molecular events responsible for immunological defects in ADA-deficient SCID; ii) the role of ADA substrates in controlling T cell survival, proliferation and functions; iii) the efficacy of gene therapy in restoring normal metabolic and immune functions.

In order to evaluate the long-term safety of HSC gene therapy, a systematic analysis of retroviral vector insertion sites has been carried out in five ADA-SCID

patients. Both inverse- and LM-PCR were set up and performed in purified hematopoietic cells subsets. The genomic impact of retroviral vector integration pattern was analyzed in terms of influence on gene expression profile at the bulk and single-cell level. Affymetrix Gene Chip arrays were used to assess expression profile in bulk population, whereas quantitative transcript analysis of genes targeted by vector integration in T-cell clones was performed by low-density gene expression array technique with two specifically designed microfluidic cards.

## **CHAPTER 2 – Materials and Methods**

### **2.1 Patients and clinical trial**

Five ADA-SCID patients have been studied in this work. Patients Pt1 and Pt2 have been previously described (Aiuti et al., 2002a) while Pt3, Pt4 and Pt5 were diagnosed as early onset ADA-SCID (<6 months of age), after showing recurrent pulmonary infections and failure to thrive. All patients were treated with gene therapy after failure of haploidentical BMT transplant (Pt2, Pt3, Pt4), or PEG-ADA treatment (Pt4, Pt5). The ADA-SCID clinical trial was approved by the H. San Raffaele Ethical Committee, the Italian Istituto Superiore di Sanità and received Orphan Drug Status by the European Medicines Agency. The patients received  $0.9$  to  $9.4 \times 10^6$  BM CD34<sup>+</sup> cells/kg transduced with an ADA-expressing MLV vector after low-dose conditioning with busulphan, as previously described (Aiuti et al., 2002a). Multilineage, stable engraftment of gene corrected stem/progenitor cells was achieved in all patients. The proportion of vector-containing cells ranged from 70 to 100% in T cells and 0.7 to 16% in granulocytes, with the exception of Pt2 who displayed low myeloid engraftment (<0.1%) (Aiuti et al., 2002a). Following gene therapy a progressive increase in lymphocyte counts, restoration of polyclonal thymopoiesis and normalization of proliferative responses to mitogens and antigens was observed in all patients. Anti-microbial prophylaxis has been either discontinued or reduced after gene therapy. Improved serum Ig levels and production of specific antibodies after IVIG discontinuation and antigen vaccination was observed in three patients. Sustained ADA activity in lymphocytes and RBC resulted in the correction of purine metabolism and amelioration of systemic toxicity. No adverse events related to gene transfer were observed in a follow-up with a median of 3.1 years (A.A. *et al.*, manuscript in preparation).

## **2.2 Cells**

Blood samples from ADA-SCID patients and healthy donors were obtained after informed consent following standard ethical procedure and with approval of the San Raffaele Scientific Institute Internal Review Board for ADA-SCID GT (Gene ADA) and healthy donors.

### **2.2.1 Purification of cell subsets from peripheral blood and BM**

Mononuclear cells from PB and BM samples were isolated by density gradient centrifugation on Ficoll-Hypaque. The PB granulocyte fraction was enriched by consecutive separations on dextran and Ficoll-Hypaque gradient. Thereafter, the different subpopulations were isolated by immunomagnetic technique using antibody-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and FACS cell-sorting using fluorescein-labelled antibodies (FACS Vantage, BD Bioscience, CA, USA). The following monoclonal antibodies were used for positive selection: anti-CD15 (granulocytes), anti-CD19 (B cells), anti-CD61 (megakaryocytes), anti-glycophorin A (erythroid cells), anti-CD56 (NK cells), anti-CD3 (T cells), anti-CD4 (CD4<sup>+</sup> T cells), anti-CD8 (CD8<sup>+</sup> T cells). Two steps of purification were performed to increase the purity to >98-99%.

### **2.2.2 T-cell lines**

Untransformed T-cell lines were generated from PBMCs purified by density gradient centrifugation on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway) and seeded at the concentration of  $0.5 \times 10^6$ /ml. Cells were stimulated with 1 µg/ml phytohemagglutinin (PHA) (Roche Diagnostics Corp.), 600IU/ml rhIL-2 (Chiron Corp., Emeryville, USA) in the presence of an allogeneic feeder mixture of  $1 \times 10^6$ /ml PBMC (X-ray irradiated at 60 Gy) and  $10^5$ /ml JY cells (X-ray irradiated at 100 Gy), an EBV-lymphoblastoid cell line expressing high levels of HLA and costimulatory molecules

(Bacchetta et al., 1994). The cultures were performed in IMDM (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% YSSEL medium (Dyaclone, Besançon, France), 5% Hyclone (Cambrex Bio Science) and 100U/ml penicillin/streptomycin (Bristol-Myers Squibb, Sermoneta, Italy). T cells were expanded by adding fresh medium with rhIL-2 and restimulated as described every 14 days. CD4<sup>+</sup> T cells subsets were purified from established T cell lines by immunomagnetic technique using anti-CD4 antibody-coated microabeads (Miltenyi Biotech, Bergische Gladbach, Germany) according to the manufacturer's instructions (purity was > 98%). All experiments shown were performed in T cell lines between day 12-15. EBV-transformed B cell line JY and was maintained in IMDM supplemented with 10 % FBS and penicillin/streptomycin.

### **2.2.3 T-cell clones**

PBMCs from Pt2 and Pt3 were isolated by Ficoll-Hypaque gradient separation and enriched for T lymphocytes by immunomagnetic-based depletion of monocytes (CD14) and B cells (CD19). Individual T-cell clones were obtained by plating cells in 96-well plates at a concentration of 0.3-1-3 cells per well. Cells were stimulated with 1 µg/ml PHA (Roche Diagnostics Corp.), 100IU/ml rhIL-2 (Chiron Corp., Emeryville, USA) in the presence of an allogeneic feeder mixture of  $1 \times 10^5$ /well PBMC (X-ray irradiated at 60 Gy) and  $10^4$ /well JY cells (X-ray irradiated at 100 Gy). The cultures were performed in IMDM (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% YSSEL medium (Dyaclone, Besançon, France), 5% Hyclone (Cambrex Bio Science) and 100U/ml penicillin/streptomycin (Bristol-Myers Squibb, Sermoneta, Italy). T-cell clones were expanded by adding fresh medium with 600IU/ml rhIL-2 and restimulated as described every 14 days.

## **2.3 Reagents**

Adenosine, 2'-deoxyadenosine, the selective A<sub>2A</sub> adenosine receptor agonist (CGS21680), the selective A<sub>2A</sub> adenosine receptor antagonist (SCH58261), the nucleoside uptake inhibitor (Dipyridamole), the adenylyl cyclase inhibitor (2',5'-Dideoxyadenosine 3'-triphosphate tetrasodium) were purchased from Sigma Aldrich (St Louis, USA). The inhibitor of cAMP-dependent protein kinase A (PKAI) was from Alexis Biochemicals (UK).

Formulation of buffers used for Western Blot: CELL LYSIS BUFFER (50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% ND-40, anti-proteases added fresh every time); LAEMMLI BUFFER (62.5 mM Tris-HCL pH 6.8; 2% w/v SDS, 10% glycerol, 5% Beta-Mercaptoethanol, Bromophenol Blu); TBS (10X: 24.2 g Tris-Base, 80 g NaCl, pH 7.6 with HCl), BLOCKING BUFFER (TBS 1X, 0.1% Tween-20, 5% BSA); TRANSFER BUFFER (250 mM Tris-Base, 2mM Glycine, Methanol final 20%).

## **2.4 Flow cytometry**

### **2.4.1 ADA expression**

Expression of ADA protein was assessed in patients and control T-cell lines with intracellular staining. Cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences Pharmingen) and incubated with anti-human ADA monoclonal antibody (kindly provided by Dr. M. Hershfield) revealed by a secondary goat anti-mouse Alexa 488 antibody (Southern Biotechnology, USA).

### **2.4.2 A<sub>2A</sub> Adenosine receptor expression**

Expression of A<sub>2A</sub> Adenosine receptor was assessed in patients' and control T-cell lines with intracellular staining. Cells were fixed and permeabilized as described above, incubated with anti-A<sub>2A</sub> Adenosine receptor (Sigma Aldrich) polyclonal antibody and revealed by goat anti-rabbit PE (Molecular Probe, Invitrogene, USA).

### **2.4.3 Apoptosis**

After time course incubation with drugs, cells were stained with Annexin V-FITC (BD Biosciences) and propidium iodide (50 µg/ml, Sigma-Aldrich).

### **2.4.4 TCR-Vbeta repertoire**

Cells ( $2 \times 10^5$ ) were stained with a mix of directly conjugated TCR Vb antibodies corresponding to 24 different specificities, according to manufactory instructions (IOtest Beta Mark kit; Immunotech, Marseille, France).

In all the experiments, the analyses were performed using FACScan flow cytometer using CellQuest software (BD Biosciences)

### **2.5 ADA and SAHH enzymatic activity**

Intracellular ADA enzyme activity was analyzed in cell lysates ( $0.5 \times 10^6$  cells) by an adenosine to inosine conversion assay, followed by high-performance-capillary-electrophoresis (HPCE) (Carlucci et al., 2003). Intracellular SAHH activity was determined in cell lysates ( $0.5 \times 10^6$  cells) by an adenosine consumption assay, followed by HPCE (Carlucci et al., 2003). Results were expressed as nmol/h/mg protein.

### **2.6 T-cell proliferation**

Resting patients' and control T-cell lines were seeded at  $1 \times 10^5$  cells/well in a 96-well plate round-bottom pre-coated with the indicated dose of anti-CD3 mAb (OKT3, Janssen Cilag, Ltd.) for 48 hours in the presence or absence of drugs with or without soluble anti-CD28 mAb (10 µg/ml; BD Biosciences), in a final volume of 200 µl. T-cell proliferation was analyzed after 48 hr of stimulation, by a 16- hours pulse with 1 µCi/well  $^3\text{H}$ -thymidine (Amersham Pharmacia Biotech) followed by harvesting using the 96-well plate harvester (Tomtec, Hamden, USA) and counting by liquid scintillation. All experiments were performed in duplicate or triplicate wells.



## **2.7 Analysis of cytokine production**

### **2.7.1 ELISA**

The secretion of cytokines was measured in the supernatants of resting and activated patients' and control T cell lines, which were plated at the initial density of  $1 \times 10^5$  cells/well. Supernatants were collected at different time points (18 hr for IL-2 and 48 hr for the other cytokines) and the presence of cytokines was detected by standard capture ELISA, which was performed according to the manufacturer's instructions (BD Biosciences Pharmingen). Concentration of cytokines was calculated by parallel measurement of a standard curve, generated using recombinant human cytokines (R&D System).

### **2.7.2 Intracellular cytokine staining**

Resting T cells ( $1 \times 10^6$ /ml) were stimulated either with coated anti-CD3 mAbs plus soluble anti-CD28 mAbs or with 10 ng/ml TPA plus 500 ng/ml ionomycin in complete medium, at 37°C, in a 5% CO<sub>2</sub> atmosphere. Upon initiation of the experiment, plates were centrifuged for 2 min at 800 rpm. After 3 hr, 10 µg/ml Brefeldin A (Calbiochem) was added to the wells. After additional 3 hr, T cells were collected and fixed in 2% formaldehyde. After fixation, T cells were permeabilized by 10 min incubation in blocking buffer (PBS 0.3% BSA, 0.1% NaN<sub>3</sub>) supplemented with 0.5% Saponin (Sigma Aldrich). Permeabilized T cells were incubated in blocking buffer with 0.5% Saponin in the presence of PE-labelled anti-hIL-4, PE-labelled anti-hIL-2 and FITC-labelled anti-hIFN-γ (BD Biosciences Pharmingen). After washing, cells were analyzed by FACS and data were analyzed by CellQuest software.

### **2.7.3 Cytokine mRNA expression**

CD4<sup>+</sup> T cells from ADA patients and healthy donors were stimulated with immobilized anti-CD3 mAb (1 µg/ml) plus soluble anti-CD28 mAb (10 µg/ml) for 5 hours. Total RNA was extracted from  $1 \times 10^6$  cells using Eurozol reagent (Euroclone

S.p.A., Milan, Italy) and reverse transcribed by using the High Capacity cDNA Archive Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Expression analysis of IL-2, IL-4 and IFN- $\gamma$  genes was carried out with TaqMan system, developed as custom "assay on demand" by Applied Biosystems. The data were analyzed by the ABI PRISM 7700 with the sequence detector system software. Samples were run in duplicate and the relative expression of cytokines was determined by calculating the difference between threshold cycles for the stimulated (target) and unstimulated (control) samples ( $\Delta$ CT method) after normalization to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT), which was run in the same tube. The results for each T cell line were expressed as n-fold difference relative to the control sample.

## **2.8 T-bet and GATA-3 mRNA expression**

To measure T-bet and GATA-3 mRNA levels, CD4<sup>+</sup> T cell lines from ADA patients and healthy donors were stimulated with immobilized anti-CD3 mAbs plus 10  $\mu$ g/ml soluble anti-CD28 mAbs for four hours. Total RNA was extracted from  $1 \times 10^6$  cells using Eurozol (Euroclone S.p.A., Milan, Italy) and RNA was reverse transcribed by using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. T-bet and GATA-3 mRNA species were quantified using the Assay on Demand kit (Applied Biosystems) and Taqman Master Mix (Applied Biosystems). Each sample was analysed in duplicate and the relative expression of T-bet and GATA-3 was defined by calculating the  $\Delta$ Ct, with respect to the housekeeping gene HPRT, run in the same tube.

## **2.9 Adenosine receptors mRNA expression**

Total RNA was isolated from CD4<sup>+</sup> T cell lines, of ADA-SCID patients and healthy donors, resting or stimulated with immobilized anti-CD3 mAb (1  $\mu$ g/ml) plus

soluble anti-CD28 mAb (10 µg/ml) for 6 hours. The RNA was reverse transcribed by using the Archive Kit (Applied Biosystems, CA, USA). Expression analysis of A<sub>2</sub>R genes was carried out using Assay-on-Demand real time PCR kits (Applied Biosystems). The expression was determined by relative quantification method. Essentially, two standard curve of serially diluted pooled cDNA, generated from resting CD4<sup>+</sup> T cell lines of 3 different NDs sample, were set up to amplify the target gene and HPRT, as endogenous control. The A<sub>2</sub>R expression in the normalized samples was expressed as n-fold difference relative to reference sample as calibrator.

## **2.10 Intracellular cAMP detection**

Bulk or CD4<sup>+</sup> T cells (2x10<sup>5</sup> cells/well) were treated with dAdo (5mmol/L) or CGS21680 (1mmol/L) with or without the inhibitor of Adenylyl cyclase (1mmol/L), in presence of 3-isobutyl-1-methylxanthine (IBMX, 1mmol/L). After 15 min incubation supernatants were decanted and cells used directly for cAMP determination, according to the manufacturer's protocol for the Direct cAMP Enzyme Immunoassay Kit (Amersham Biosciences). The assay was performed in duplicate wells.

## **2.11 Western Blotting**

### **2.11.1 ADA expression**

Cell extracts were prepared from 1x10<sup>6</sup> resting T cells. Briefly, T cells were washed in PBS and resuspended in lysis buffer supplemented with protease inhibitor cocktail (Sigma Aldrich, St-Louis, USA). After 30 min on ice, lysates were cleared by centrifugation and an aliquot of supernatants was used for determining protein concentration with the BCA protein assay (Pierce, Rockford, USA). Laemmli buffer was added to supernatants and, after boiling, equal amounts of proteins were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane and ADA was detected by the anti-ADA monoclonal Ab (kindly provided by Dr. Hershfield, Duke University Medical Center, Durham, NC) followed by secondary HRP-conjugated anti-

mouse Abs (Dako Cytomation). Control of protein loading was performed by hybridizing the same membranes with an anti-G3PDH mAb (Chemicon, Temecula, CA, USA). Detection was performed using ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

### **2.11.2 Analysis of ERK1/2 phosphorylation**

CD4<sup>+</sup> T cell lines from ADA patients and from healthy donors were starved for 12 hours in IMDM containing low fetal bovine serum (1%), then stimulated, in the same medium supplemented or not with dAdo (0.5 mmol/L), by TCR cross-linking with 1 µg/ml anti-CD3 mAb plus 10 µg/ml anti-CD28 mAb for the indicated times. Reactions were stopped by wash in cold PBS and immediate lysis in whole-cell lysis buffer. After 30 min on ice, lysates were cleared by centrifugation and an aliquot of supernatants was used to determine protein concentration with the Bradford protein assay (Bio-Rad). Laemmli buffer was added to supernatants and, after boiling, samples with equal amount of proteins were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane and phosphorylated ERKs were detected by anti-phosphoERK1/2 mAb (Cell Signaling Technologies, Inc., Danvers, MA, USA). To detect levels of total ERK1/2, membranes were stripped by a 15-min incubation, at room temperature, in Restore Buffer (Pierce Biotechnology, Rockford, USA) and re-probed with anti-ERK1/2 pAb (Cell Signaling Technologies, Inc.). Incubation with primary Abs was followed by incubation with goat anti-mouse or anti-rabbit HRP Ab, according to the specificity of the primary Abs (Dako Cytomation). Detection was performed by ECL detection system (Amersham Pharmacia Biotech).

### **2.11.3 Analysis of CREB and IκBα phosphorylation**

CD4<sup>+</sup> T cells from ADA patients and healthy donors were starved for 12 hours in low serum (1%), then stimulated by TCR cross-linking with 1 µg/ml anti-CD3 plus 10 µg/ml anti-CD28 for the indicated times. Reactions were stopped by wash in cold

PBS and immediate lysis in whole-cell lysis buffer. After 30 min on ice, lysates were cleared by centrifugation and an aliquot of supernatants was used to determine protein concentration with the Bradford protein assay (Bio-Rad). Laemmli buffer was added to supernatants and, after boiling, samples with equal amount of proteins were subjected to SDS-PAGE. Upon blotting onto nitrocellulose membrane, levels of phosphorylated CREB were detected by anti-phosphoCREB mAb (Cell Signaling Technology, Inc.), followed by membrane re-probing with Total-CREB pAb (Cell Signaling Technology, Inc.). Phosphorylation levels of I $\kappa$ B $\alpha$  were detected by anti-phosphoI $\kappa$ B $\alpha$  (Cell Signaling Technology, Inc.). Control of protein loading was performed by hybridizing the same membranes with anti-G3PDH Ab.

## **2.12 DNA purification and quantitative PCR for vector positive cells**

Genomic DNA was extracted from T cells using QIAamp DNA Blood Mini kit (Qiagen Inc. Valencia, California). Quantitative PCR analysis for vector positivity was performed as previously described (Aiuti et al., 2002b). Briefly, two sets of primers and probes specific for Neo<sup>R</sup> gene and GAPDH were used to detect transduced cells and standardize for DNA content, respectively. The frequency of transduced cells was calculated using a standard curve and expressed as the proportion of cells containing the Neo<sup>R</sup> reporter gene.

## **2.13 Analysis of retroviral vector integration site.**

In order to avoid potential biases related to the use of restriction enzyme or amplification steps, two different techniques were used for analysis of integration sites: the inverse (I-) and linker-mediated (LM-) PCR.

### **2.13.1 Inverse-PCR**

I-PCR was performed by a modification of previously described primers and PCR conditions (Kim et al., 2000). Briefly, 50 ng of genomic DNA were digested with TaqI and the LTR-containing fragments self-ligated and subjected to two rounds of

amplification using the following LTR-specific primers: Forward: 5'-CTGTTTCCTTGGGAGGGT-3'; Reverse: 5'-AGGAACTGCTTACCACA-3'; nested forward: 5'-GCGTTACTTAAGCTAGCTTG-3'; nested reverse: 5'-GATTGACTACCCACGACGGG-3'. Reaction conditions were identical for the first and second round of PCR: 94° denaturation for 1 min, 50° annealing for 1 min, 72° extension for 1.5 min, for a total of 29 cycles.

### **2.13.2 LM-PCR**

For LM-PCR, 200 ng of genomic DNA were digested with *Pst*I and *Mse*I to prevent amplification of an internal viral fragment from the 5'LTR, and ligated to an *Mse*I double-strand linker. Nested PCR was performed with LTR- and linker-specific primers as previously described (Wu et al., 2003).

### **2.14 PCR products cloning**

The resultant PCR products were shotgun-cloned without purification with the PCR 2.1 TOPO TA cloning kit (Invitrogen, CA, USA) and transformed into libraries of vector-integration junctions. Alternatively, nested PCR products were separated either on 2% agarose or on Spreedex gels (Elchrom Scientific, Switzerland), excised and directly sequenced (Primm s.r.l., Italy). Mapped integrations were selected for analysis only if contained the correct LTR- and primer-specific sequences and yielded a unique best hit with  $\geq 95\%$  of identity to the human genome.

### **2.15 Integrants-specific Q-PCR**

To determine the relative contribution of *LMO2*-related molecular clones over time, real-time Q-PCR were set up to specifically amplify the proviral-genomic junction of integrations within the *LMO2* locus (S1\_049, S4\_048, S3\_042 and S5\_144). Two molecular clones isolated from Pt3 T cells mapping to “neutral” regions of the genome (S3\_082 and S3\_116), were used as control of physiological clonal fluctuations. The Q-PCR analysis was performed on 50 ng of genomic DNA, directly isolated from sorted

cells, using the specific unique genomic flanking primers in combination with common LTR primer and probe (Primm s.r.l.). The primers sequence was as follows: LTR Primer: 5'-GTTTGCATCCGAATCGTGGT-3';

LTR probe: 6-FAM-TCTCCTCTGAGTGATTGACTACCCACGACG-TAMRA;

S1\_049 Primer: 5'-GGAATCAGGCACCTTCTCTCTC-3';

S4\_048 Primer: 5'GGTTAAGATCACAGGCTGTGGTG-3';

S3\_042/S5\_144 Primer: 5'-TCTCTCCTATCAGCCAATAAAGGG-3';

S3\_082 Primer: 5'-CGGCTCGGGTGTCTCTG-3';

S3\_116 Primer: 5'-AAGTGTCGGTGTTAGTACCC-3'.

Each sets of primers allowed to unambiguously track the corresponding clone, as assessed by the absence of amplification in samples from different patients. The frequency of each individual clone, expressed as the proportion of cells containing the specific vector sequence, was calculated on the basis of a standard curve of the relative integrants diluted in untransduced PB lymphocytes (from  $5 \times 10^4$  to  $5 \times 10^0$ ).

## **2.16 RNA purification and retrotranscription**

Total RNA was isolated from CD3<sup>+</sup>, CD4<sup>+</sup> or CD8<sup>+</sup> T cells as well as CD15<sup>+</sup> granulocytes purified either from GT-treated patients, untreated ADA-SCID patients or age-matched healthy donors. The RNA was reverse transcribed by using the Archive Kit (Applied Biosystems, CA, USA).

## **2.17 RT- PCR analysis for *LMO2* expression**

Expression analysis of *LMO2* gene was carried out with TaqMan system, developed as custom "assay on demand" by Applied Biosystems. The data were analyzed by the ABI PRISM 7700 with the sequence detector system software. *LMO2* expression was determined by relative quantification method. Essentially, two standard curve of serially diluted cDNA, obtained from one healthy donor granulocytes sample, were set up to amplify the target gene and HPRT, as housekeeping endogenous control.

The *LMO2* expression in the normalized samples was expressed as n-fold difference relative to reference sample as calibrator.

## **2.18 Microarray: Affymetrix HG-U133A Gene Chip Array**

RNA was isolated from BM-derived CD34<sup>+</sup> cells (n=2) cultured ex vivo according to the cytokine conditions used in the clinical protocol, and from primary CD3<sup>+</sup> T cells (n=5) or CD4<sup>+</sup> T cells (n=8). The transcribed biotinylated cRNA, was cleaned up using RNeasy spin columns (Affymetrix), fragmented and hybridized to an Affymetrix HG-U133A Gene Chip Array. The scanned images were analyzed using the Affymetrix MAS 5.0 suite, and the signal intensity of detected transcripts (probesets) was determined with the MAS 5.0 absolute analysis algorithm. Following inter-array normalization of overall fluorescence intensities, the differences in gene expression levels were expressed as numeric values. In the correlation between integrations and gene activity, the expression values of each probeset classified as a “present” by the software, were assigned to three arbitrary classes corresponding to 0-25<sup>th</sup>, 25<sup>th</sup> -75<sup>th</sup> and 75<sup>th</sup> -100<sup>th</sup> percentile of expression level of all present genes (probesets) in a normalized data distribution.

## **2.19 Taqman Low Density Array**

cDNA were reverse transcribed according to the Archive Kit (Applied Biosystems) manufacturing instructions. Taqman PCRs were carried out onto custom 7900 Taq-man low density arrays on an ABI PRISM 7900 HT (Applied Biosystems). Taqman strategies for each gene were developed as “Assay on Demand” by Applied Biosystems. Gene expression profiling was achieved by using the comparative cycle of threshold (CT) method for relative quantification. For each detector, in the normalized sample, the  $\Delta\Delta C_t$  expression was calculated by using the average of  $\Delta C_t$ s in all samples as calibrator.



## 2.20 Functional clustering analysis

Probesets corresponding to genes targeted by retroviral vector insertions were analyzed for functional clustering into specific categories according to Gene Ontology criteria. The probability of over-representation of each category was determined using the EASE bioinformatics package (<http://david.niaid.nih.gov/david/ease.htm>). The default metric used by EASE to rank categories of genes by over-representation provides the EASE score.

## 2.21 Bioinformatics

The bioinformatic analysis was performed by dedicated Perl scripts allowing the storage, retrieval, analysis and summary of the data through a database. Human RefSeq chromosome sequence FASTA files for the May 2004 human genome freeze (NCBI v.35) were downloaded from the NCBI genome project website and converted to be used in local Blast search. Coordinates of RefSeq genes and other annotation tables for the same genome reference sequence freeze were accessed through the Ensembl Perl API from the Ensembl genome project. In the present study, we assumed the definition of “gene” as the genomic region between the transcriptional start site and stop boundaries of one of the 24,194 RefSeq genes mapped to the human genome, as reported by Wu et al.

## 2.22 Statistics

A two-tailed Mann Whitney  $U$  test was used for the analysis of statistically significant differences comparing two independent groups of individuals, ADA-SCID patients versus healthy controls. Responses within the same individual were compared by the (two tailed) Wilcoxon signed rank test for paired data. Comparisons between *in vitro* and *ex vivo* RIS frequency were tested with a Pearson Chi-Square test. In the gene expression studies, given the different sample sizes to account for the difference distribution between each sample and an asymptotic approximation was considered via

the Monte-Carlo method based on 50.000 or 100.000 replications. Statistical significance was evaluated by a parametric likelihood test, distributed as a Chi-square. Analysis of overrepresentation of probesets classes in the integration data set was performed by means of both Fisher's exact test as well as Easy Score (DAVID-EASE software). The results were corrected for multiple testing errors within each data set/system combination with Holm and Bonferroni correction methods.

## **CHAPTER 3 - Results**

### **3.1 Generation of untransformed T-cell lines from ADA-SCID patients**

Primary ADA-deficient T lymphocytes are difficultly available for experimental investigations since patients affected by ADA-SCID are severely lymphopenic. Most of information about the role ADA in the biochemical and cellular metabolism of human ADA-deficient cells has been derived from studying either ADA-deficient EBV-transformed B-cell lines and Herpes Virus-transformed T-cell lines (Otsu et al., 2002) (Ariga et al., 2001). However, different parameters, such as ADA activity, might not be faithfully reflected in virus-transformed cell lines. Moreover, the immunological defect was incompletely investigated. We successfully proceeded to *in vitro* expansion of untransformed T-cell lines from five patients (Table 1), before (ADA<sup>-/-</sup>) or 6 to 48 months after gene therapy (GT) with autologous genetically-corrected hematopoietic stem/progenitor cells (Illustration XVII). The culture protocol used (see methods section) allowed to growth T-cell lines quite long-term without any alteration in their original metabolic and immune phenotype. Indeed, in agreement with what we have observed analyzing Vbeta TCR repertoire in whole blood, while ADA<sup>-/-</sup> cell lines display a more oligoclonal profile, T-cell lines generated after GT have a polyclonal pattern (data not shown), which were maintained throughout the culture period. The percentage of vector-positive cells, as detected by qPCR, ranged between 87-100% in GT cell lines (Aiuti et al., 2002a) (Aiuti A. et al., *manuscript in preparation*).

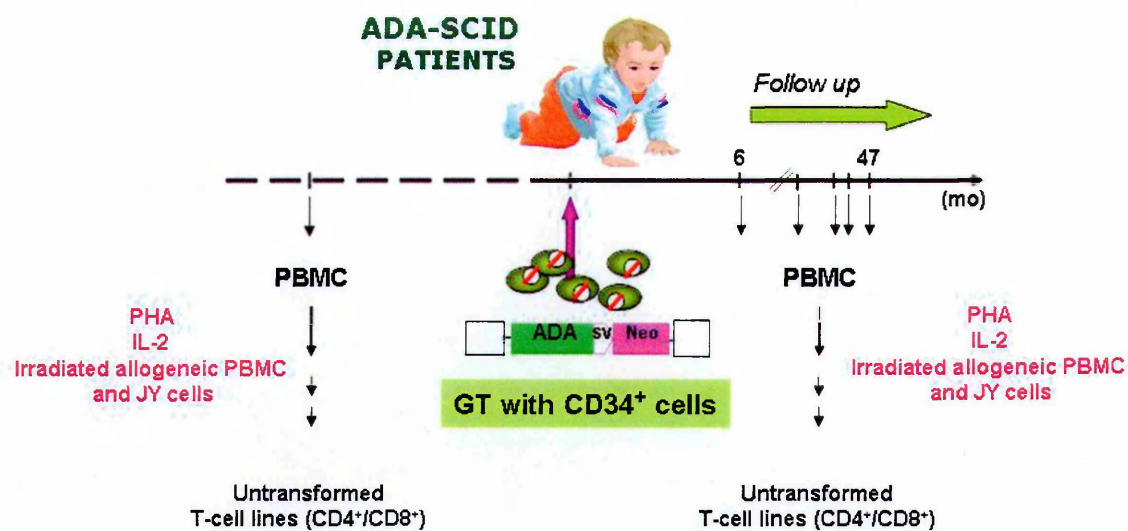


Illustration XVII. Experimental model.

Patient	nt Change	Site	aa Change	Zygosity
<b>Pt1</b>	CAC-CCC	exon 2	His17Pro	homozygous
<b>Pt2</b>	CTG-CCG	exon 4	Leu107Pro	heterozygous
	CGT-CAT	exon 7	Arg211His	heterozygous
<b>Pt3</b>	GGC-GTC	exon 4	Gly74Val	heterozygous
	GGC-GAC	exon 10	Arg282Gln	heterozygous
<b>Pt4</b>	GGC-GAC	exon 10	Arg282Gln	homozygous
<b>Pt5</b>	GGG-AGG	exon 7	Gly216Arg	heterozygous
	del AGA	exon 10	Glu319fsX3	heterozygous

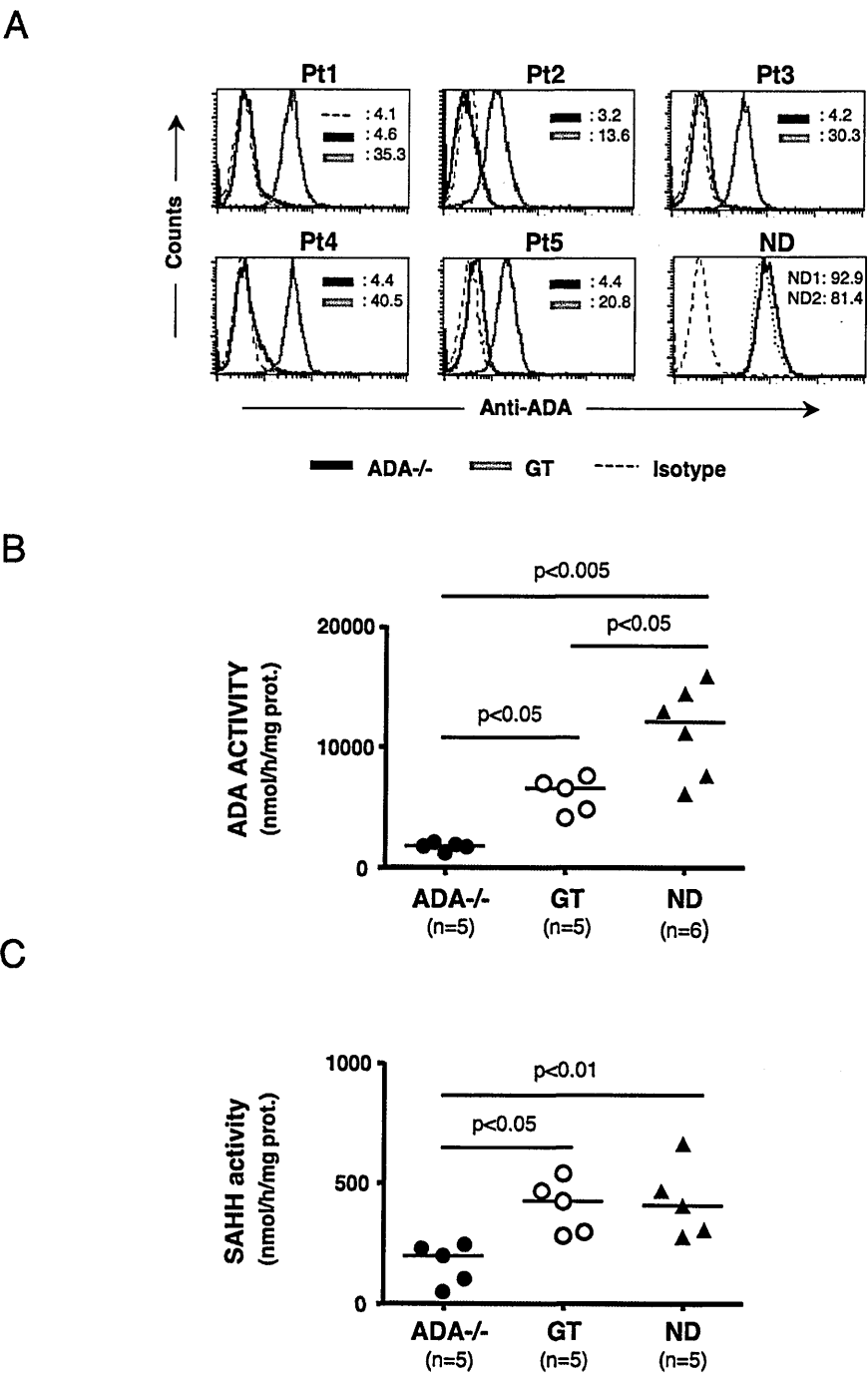
Table 1. Mutations of ADA-SCID patients.

### **3.2 Restoration of ADA expression after gene therapy corrects the SAHH defect in ADA-deficient T cells.**

In order to investigate the effect of mutations in ADA gene on protein stability and enzyme activity, ADA protein expression was analyzed by intracytoplasmic staining in T cells from patients and age-matched healthy controls (ND) after short-term culture in FBS-free medium, to avoid the potential interference of the bovine ADA in the analysis. ADA expression is completely absent in ADA<sup>-/-</sup> bulk T cells whereas it is restored in GT cells, displaying an intermediated ADA phenotype with respect to the controls (Fig. 1A). In agreement, a stronger ADA enzymatic activity was measured in GT cells as compared to ADA<sup>-/-</sup> cells (Fig. 1B,  $p < 0.05$ ), demonstrating that in these cells the enzyme was expressed but, more importantly, functional.

The metabolic consequences associated with the ADA-deficiency have been implicated in the irreversible intracellular inactivation of the enzyme S-adenosylhomocysteine hydrolase (SAHH) in the erythrocytes of untreated ADA-deficient children (Hershfield, 2001). This secondary deficiency has been pointed out as an additional hallmark of the disease. Interestingly, a markedly reduced SAHH activity was found in ADA<sup>-/-</sup> T cells compared to NDs (Fig. 1C,  $p < 0.01$ ), indicating that an intrinsic defect in the purine metabolism persist in ADA<sup>-/-</sup> cells even when cultured *ex-vivo* in potential toxins-free conditions. Remarkably, SAHH activity was completely restored at normal level in GT cells, suggesting that in these cells the transgene expression is at therapeutic level, enabling the full correction of the purine metabolic defect.

Figure 1



**Figure 1. Restored ADA and SAHH activity in T cells from ADA-SCID patients after gene therapy.** (A) Bulk T-cell lines established from five ADA-SCID patients, before (black line) and after HSC gene therapy (grey line), and from healthy controls were cultured for four days without serum prior to intracytoplasmic labeling of ADA. M.F.I. values are indicated in the histogram plots. (B) ADA activity and (C) SAHH activity were measured in T-cell lines ( $1 \times 10^6$  cells) as described in the methods section.

### **3.3 Characterization of the defective T-cell functions in ADA<sup>-/-</sup> T cells**

#### **3.3.1 ADA<sup>-/-</sup> T cells show defective proliferation in response to TCR/CD28-mediated stimulation**

Our T cell cultures contain a mixed population of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, with a preferential expansion of CD4<sup>+</sup> T cells in cultures (not shown). These two cell subsets can have intrinsic differences in their response to anti-CD3 mAb stimulation, which could derive from different threshold required for activation, response to co-stimulation or even susceptibility to activation induced cell death (AIDC). In addition, it is possible that in a mixed culture the response of one cell subset is influenced by the other one, by expression of membrane molecules or by release of soluble factors. Therefore, we decided to perform experiments using purified CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines, in order to clarify their relative contribution in the immune defect.

Upon activation with escalating doses of plate-bound anti-CD3 mAb, which is known to provide a strong TCR-mediated stimulation, the proliferative responses of purified ADA<sup>-/-</sup> CD4<sup>+</sup> T cells were severely impaired, irrespectively of the dose of mAb used ( $p < 0.01$ , Fig. 2A). In contrast, normal proliferative responses were observed in T-cell lines derived ex-vivo from patients treated with gene therapy (Fig. 2A). Similar results were obtained with CD8<sup>+</sup>-enriched T-cell lines (purity > 85%) of both patients and healthy controls (data not shown). The addition of anti-CD28 mAb (10 µg/ml) enhanced the proliferative ability of ADA<sup>-/-</sup> T cells without achieving the normalization (data not shown).

#### **3.3.2 Defective production of cytokines by ADA<sup>-/-</sup> CD4<sup>+</sup> T cells upon TCR/CD28 triggering**

A defective proliferation could result, at least in part, from weak IL-2-driven signals delivered to T cells. To determine whether a sub-optimal IL-2 secretion could be

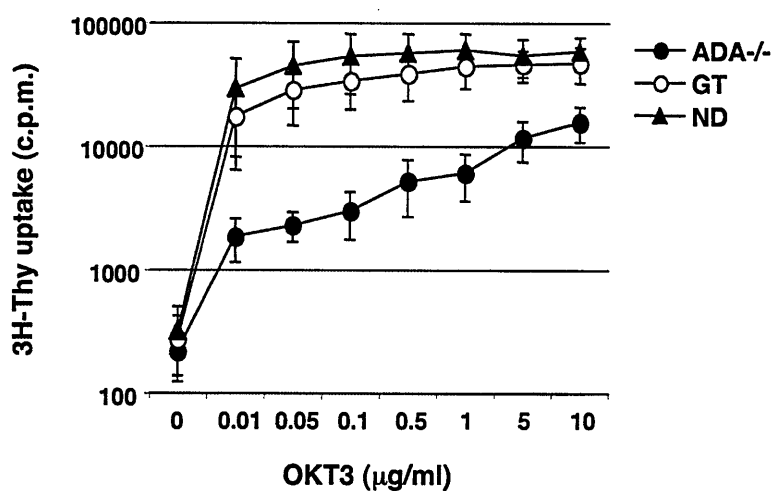
primarily linked to the defect observed in ADA<sup>-/-</sup> cells, we measured the release of IL-2 in the supernatants of anti-CD3 stimulated cells. As shown in Fig. 2B, ADA<sup>-/-</sup> CD4<sup>+</sup> T cells released 2-log less IL-2 compared with cells from healthy controls (p<0.01). Interestingly, the restored IL-2 secretion in GT CD4<sup>+</sup> cells (p<0.05 vs ADA<sup>-/-</sup>) paralleled the correction of the proliferative responses (Fig. 2A-B). To investigate whether the deficient cytokine secretion in ADA<sup>-/-</sup> cells was IL-2-specific or also affected other cytokines, we further analyzed the cytokine profile. Secretion of IFN- $\gamma$ , TNF- $\alpha$  and IL-10 was severely compromised in ADA<sup>-/-</sup> CD4<sup>+</sup> cells (Fig. 2B, p<0.01 vs NDs). In contrast, the defect in IL-4 and IL-5 secretion was less pronounced, even if still present (Fig. 2B, p<0.05). These data were confirmed when production of IL-2, IFN- $\gamma$  and IL-4 was measured at single cell level in CD4<sup>+</sup> T cells. Upon TCR/CD28 triggering, the percentages of IL-2 and/or IFN $\gamma$ -producing cells were strongly reduced in ADA<sup>-/-</sup> CD4<sup>+</sup> cells with respect to healthy donors (p<0.01; Fig. 2C and Table 2). In particular, the population of cells producing IFN- $\gamma$  was predominantly affected. The production of IL-4 by ADA<sup>-/-</sup> CD4<sup>+</sup> T cells was higher compared to other cytokines but still below the control range (p<0.05; Fig. 2C and Table 2). However, the cells producing both IL-4 and IFN- $\gamma$  were almost completely absent in ADA<sup>-/-</sup> cells. Stimulation with TPA and calcium ionophore, which bypass the TCR, resulted in significantly higher production of all cytokines but confirmed the defective production of IFN- $\gamma$  (p<0.05; Fig. 2C and Table 2). Collectively, these data demonstrate that in ADA-deficient CD4<sup>+</sup>T cells, defective cytokine secretion is the consequence of their blocked production. Moreover, there is also clear indication that, although not specifically restricted, the impaired anti-CD3/anti-CD28 response in ADA<sup>-/-</sup> cells is particularly affecting the Th1-type cytokines. In contrast, in gene-corrected CD4<sup>+</sup> T cells the production of both Th1- and Th2-like cytokines was significantly improved



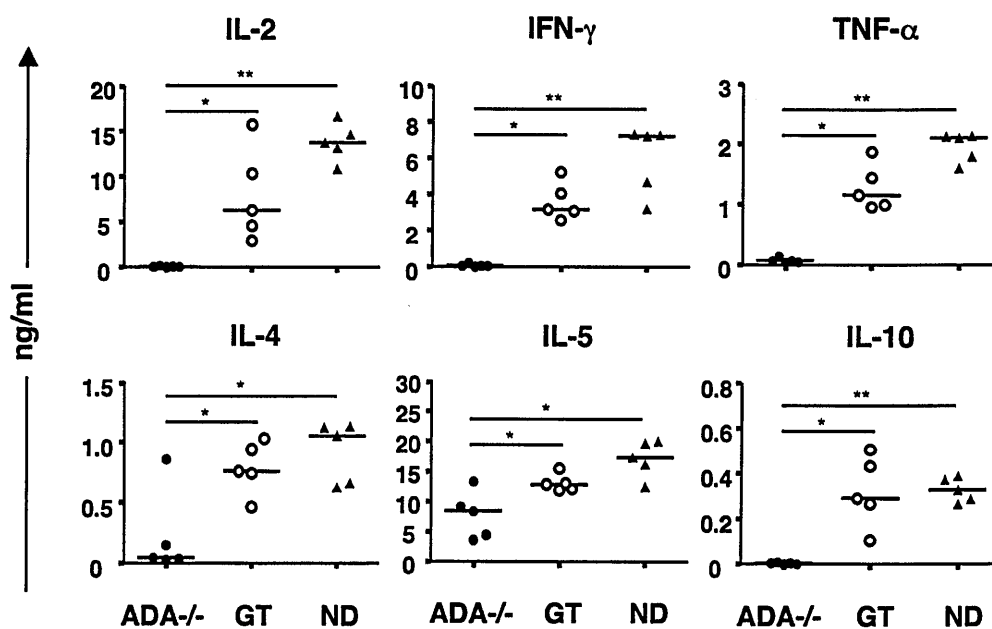
compared to deficient cells ( $p < 0.05$ ), reaching the range of controls (Fig. 2C and Table 2).

Figure 2

A



B



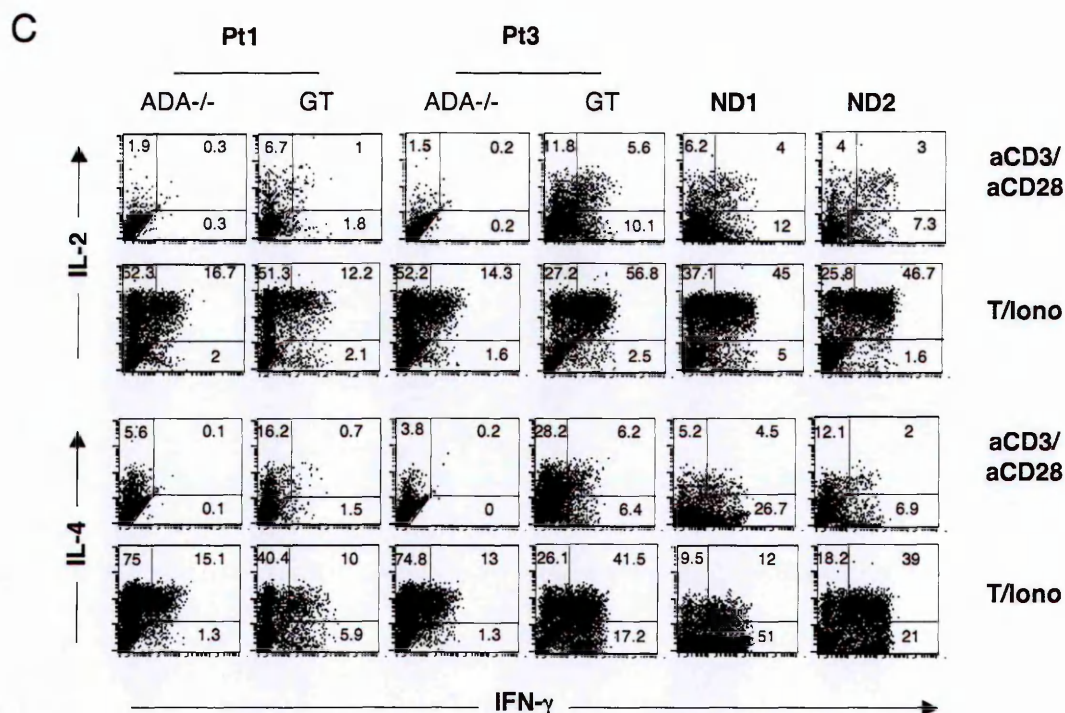


Table 2.

	aCD3/aCD28			TPA/Iono		
	IL-2+ cells (%)	IL-4+ cells (%)	IFN- $\gamma$ + cells (%)	IL-2+ cells (%)	IL-4+ cells (%)	IFN- $\gamma$ + cells (%)
ADA <sup>-/-</sup> (n=5)	1.18 - 1.85**	3.6 - 5.6*	0.14 - 1.23**	66.1 - 72.4	44.4 - 89.7	14.6 - 37.1*
GT (n=5)	7.4 - 24.8	10 - 29.2	2.8 - 23.2	63.5 - 89.4	33.1 - 67.4	14.3 - 59.2
ND (n=7)	9.07 - 16.04	4.7 - 15.6	9.5 - 15.5	31.1 - 82.1	21.4 - 58.2	16.7 - 58.2

\* p<.005 vs ND

\*\* p<.001 vs ND

**Figure 2. Characterization of the T-cell effector functions defect in CD4<sup>+</sup>ADA-deficient T cells.** (A) Purified CD4<sup>+</sup> T cell lines from five patients and five healthy controls were stimulated with immobilized anti-CD3 mAb at the indicated concentrations and [<sup>3</sup>H]thymidine incorporation was measured after 48 hours. (B) Supernatants were collected after 18 (IL-2) or 48 hours (all other cytokines) in cells stimulated with 1  $\mu$ g/ml of anti-CD3 and cytokine concentrations were quantified by capture ELISA. Each value represents the mean of cytokine concentration measured in triplicate cultures for each patients and healthy controls. Median values are also reported. Background levels were subtracted. One determination out of 3 is shown. (C) Production of IFN- $\gamma$  vs either IL-2 or IL-4, was analyzed by intracytoplasmic staining in CD4 T-cell lines from ADA-SCID patients and healthy donors following 6 hours stimulation with immobilized anti-CD3 (1 $\mu$ g/ml) plus anti-CD28 (10  $\mu$ g/ml) mAbs, or with TPA plus ionomycin. The experiment shown is representative of 5 independent experiments. Two ADA-SCID patients and one healthy donor are reported. Range values corresponding to five patients and seven NDs are indicated in the Table 2.

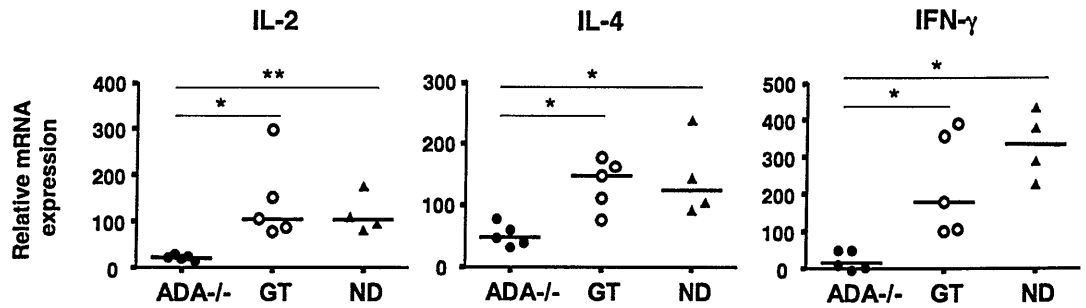
### 3.3.3 Defective cytokine production reflects impaired cytokine gene transcription in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells

As a result of TCR engagement, activation-specific genes are induced by binding of multiple transcription factors within the promoter regions. Among them, cytokine genes are the most acutely transcribed (Hughes-Fulford et al., 2005), because of their key role in sustaining the following phases of cell proliferation and differentiation. To investigate whether defective transcription of cytokine genes could account for their impaired production in ADA-deficient cells, we analyzed by Real-Time PCR the cytokine mRNA levels in CD4<sup>+</sup> T cells from five ADA-SCID patients and four healthy controls.

Upon TCR/CD28-dependent activation, the overall levels of IL-2, IL-4 and IFN- $\gamma$  expression were significantly lower in ADA<sup>-/-</sup> cells compared to those detected in NDs (Fig. 3;  $p < 0.01$  for IL-2;  $p < 0.05$  for IL-4 and IFN- $\gamma$ ) or in post-GT cells ( $p < 0.05$ ).

Interestingly, ADA<sup>-/-</sup> CD4<sup>+</sup> T cells reproducibly showed higher IL-4 *versus* IFN- $\gamma$  mRNA ratio than control CD4<sup>+</sup> T cells, indicating a difference in the balance between these two cytokines. Stimulation with TPA and calcium ionophore, which bypass the TCR, confirmed the Th1 biased cytokine mRNA profile for ADA<sup>-/-</sup> CD4<sup>+</sup> cells (data not shown), but in contrast to TCR/CD28 stimulation, the differences with respect to control cells did not reach the statistical significance. Overall these results indicate that the cytokine defect is present at the transcriptional level after TCR-dependent activation.

Figure 3



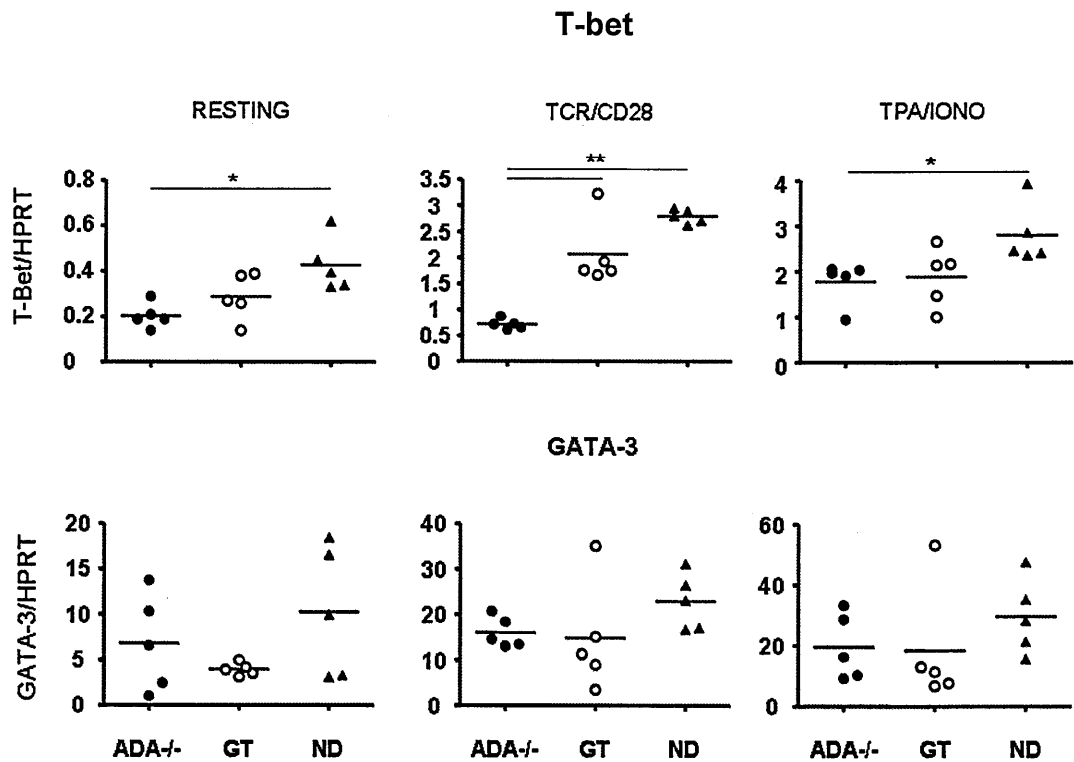
**Figure 3. Analysis of cytokine mRNA expression in CD4<sup>+</sup> T-cell lines.** (A) Measurement of cytokine gene expression after TCR/CD28-mediated stimulation of CD4<sup>+</sup> T-cell lines from five patients and four healthy donors. Total RNA was prepared from either resting or stimulated cells (1 μg/ml anti-CD3 plus 10 μg/ml anti-CD28 mAbs) and gene expression was measured by semi-quantitative RT-PCR. Values were normalized for the expression of the housekeeping gene HPRT and correspond to duplicate samples. Results are expressed as n-fold difference relative to resting condition. One representative experiment out of three is shown. \*p<0.05; \*\*p<0.01

### 3.4 Reduced T-bet mRNA induction in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated via TCR/CD28

We next analyzed the levels of T-bet and GATA-3 mRNA in ADA<sup>-/-</sup> CD4<sup>+</sup> T cell lines, as these transcription factors are involved in the transcription of IFN-γ and IL-4 genes, respectively (Szabo et al., 2002) (Zheng and Flavell, 1997). Level of T-bet mRNA was lower in resting ADA<sup>-/-</sup> CD4<sup>+</sup> T cells compared to healthy controls (p<0.05; Fig. 4, upper panel). Upon stimulation with 1 μg/ml anti-CD3 plus 10 μg/ml anti-CD28 mAbs, T-bet mRNA expression was significantly higher in control and gene-corrected cells than in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells (p<0.01; Fig. 4, upper panel). Stimulation with TPA plus ionomycin confirmed the defective T-bet expression in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells (p<0.05; Fig 4, upper panel). In contrast, levels of GATA-3 mRNA were comparable in resting and activated CD4<sup>+</sup> T cells from ADA-SCID patients and healthy controls (Fig. 4 lower panels).

These data show that ADA-deficient CD4<sup>+</sup> T cells have a reduced ability to induce T-bet mRNA upon activation. Moreover, reduced T-bet expression in resting may be reflective of a defect in T-cell development.

Figure 4



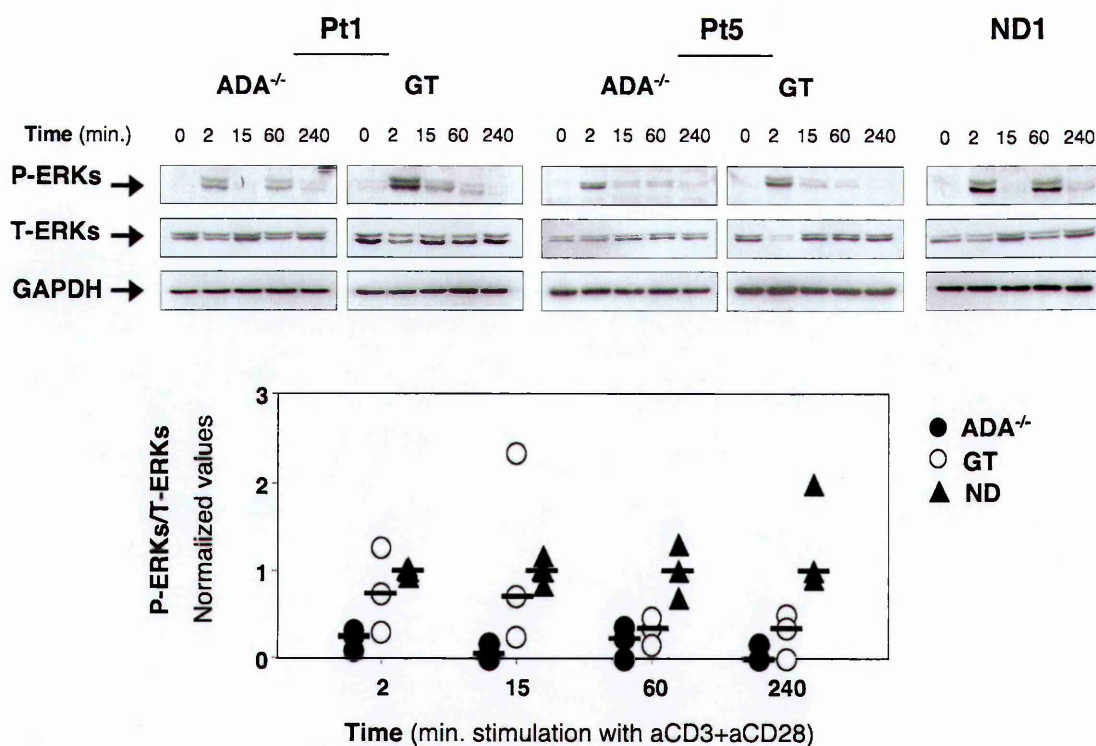
**Figure 4. Analysis of T-bet and GATA-3 relative mRNA levels in resting and activated CD4<sup>+</sup> T-cell lines from ADA-SCID patients.** Measurement of T-bet (upper panels) and GATA-3 (lower panels) gene expression before and after stimulation of CD4<sup>+</sup> T cells from five ADA-SCID patients and five healthy controls. Total RNA was prepared from either resting or stimulated cells and gene expression was measured by semi-quantitative RT-PCR. Values were normalized on expression of the housekeeping gene HPRT and correspond to duplicate wells. Results derived from one out of three independent experiments are shown. Statistical significance is represented as follows: \*  $p < 0.05$ ; \*\*  $p < 0.01$

### **3.5 Biochemical basis for impaired effector function of ADA<sup>-/-</sup> T cells**

#### **3.5.1 Reduced ERK1/2 phosphorylation in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells**

Since the impaired effector functions, in ADA-deficient cells, was proved to be strictly dependent on TCR engagement, we investigated whether it could result from poor activation of ERKs signaling, the main pathway integrating TCR-activating signals in mature T cells (Rincon et al., 2001). Stimulation of control CD4<sup>+</sup> T cells, with high dose of cross-linked anti-CD3 mAb and anti-CD28 mAb, lead to a strong induction of ERK1/2 phosphorylation, peaked as early as 2 min, and remaining detectable for later time points before waning at 240 min (Fig. 5). In ADA<sup>-/-</sup> CD4<sup>+</sup> T cells ERKs phosphorylation was consistently below the threshold of activation detected for the healthy controls, suggesting that an intrinsic reduced T-cell activation underlies the observed hypo-responsiveness. Interestingly, in GT CD4<sup>+</sup> T cells ERKs phosphorylation occurred properly at 2 and 15 min after the TCR cross-linking, and was maintained, to a lesser extent, throughout the time course (Fig. 5).

These data, showing that activation of ERK1/2 is defective in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells, indicate that the signaling leading to the activation of these molecules, downstream of TCR/CD28, is also likely impaired in ADA-deficient T cells.



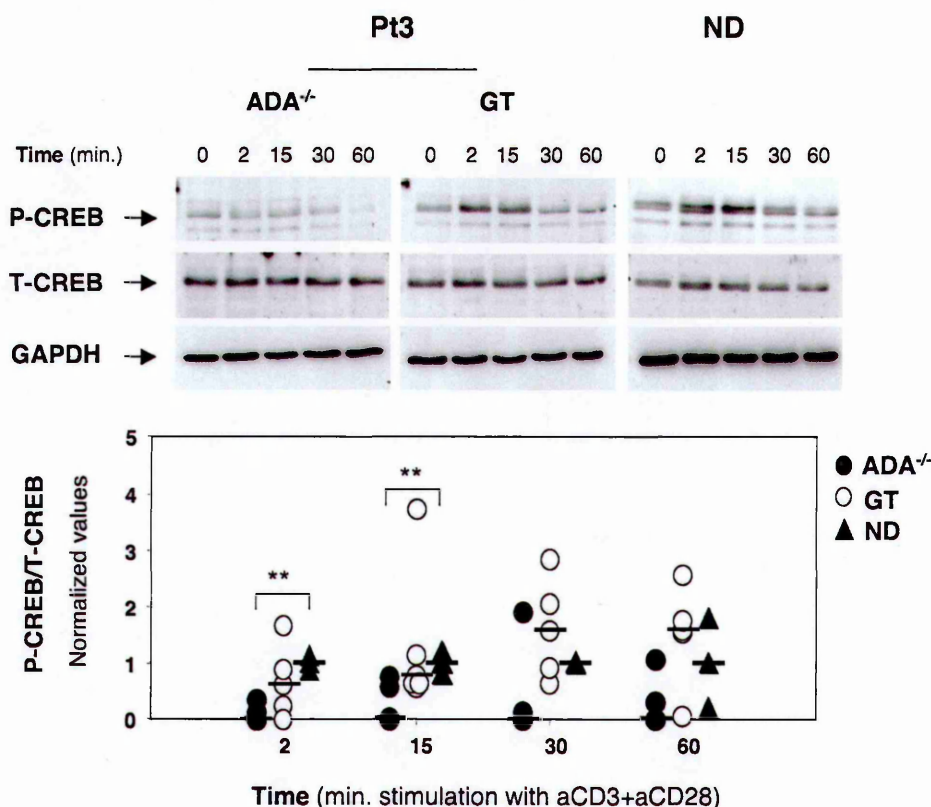
**Figure 5. Analysis of ERK1/2 phosphorylation in CD4<sup>+</sup> T-cell lines from ADA-SCID patients.** The levels of ERK1/2 phosphorylation were analyzed by Western blot in CD4<sup>+</sup> T-cell lines from patients and healthy donors following stimulation with cross-linked 1 $\mu$ g/ml anti-CD3 plus 10  $\mu$ g/ml anti-CD28 mAbs for the indicated time points. After blotting with anti-phospho-ERKs the membranes were stripped and re-probed with anti-total-ERKs and anti-GAPDH for control of protein loading. Upper panel: results of Western blot analysis are shown for Pt1 and Pt5 and for one healthy donor. Lower panel: densitometric analysis on three patients and three NDs is reported. Values corresponding to unstimulated cells were subtracted.

### 3.5.2 Defective CREB phosphorylation in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells

In previous experiments, we observed a defective transcription of cytokine genes in ADA-deficient T cells from patients. Since these findings mirrored a sub-optimal induction of the transcriptional machinery, we examined the status of activation a early transcription factor, which is mandatory for the transcription of IL-2 and other cytokine genes, CREB (Hughes-Fulford et al., 2005). Upon TCR engagement, CREB is rapidly phosphorylated on Ser133, resulting in its activation (Johannessen et al., 2004). Patients and control CD4<sup>+</sup> T cells were stimulated with cross-linked anti-CD3 plus anti-CD28



mAbs, and CREB phosphorylation was analyzed by western blot. As expected, upon TCR triggering, CREB was rapidly (2 min.) phosphorylated/activated in normal T cells, returning to basal level within 30-60 min of stimulation (Fig. 6). In contrast, early CREB activation was strongly impaired in ADA<sup>-/-</sup> cells from all five patients tested (Fig 6, p<0.01 vs NDs.). Remarkably, this pre-transcriptional event occurred normally in GT T cells.

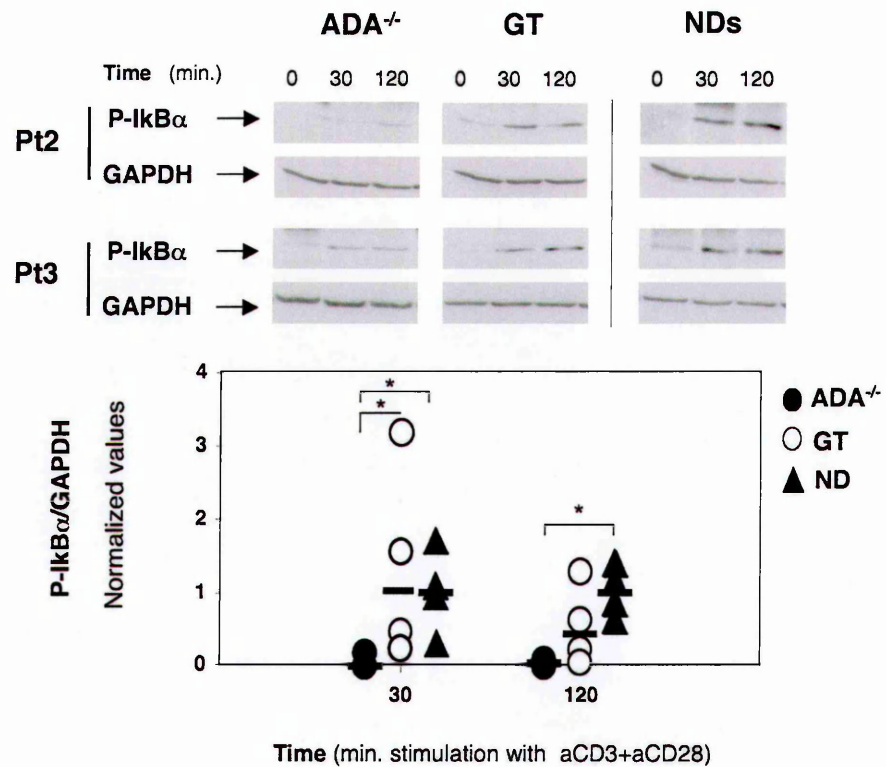


**Figure 6. Analysis of CREB phosphorylation in CD4<sup>+</sup> T-cell lines from ADA-SCID patients.** Immunoblots depicting phosphorylated CREB, total CREB and GAPDH are shown. CD4<sup>+</sup> T-cell lines were stimulated with cross-linked (1μg/ml) anti-CD3 plus (10 μg/ml) anti-CD28 mAbs for the indicated time points. The same membranes were sequentially probed for the different Abs. Upper panel: a representative experiment in Pt3 and one healthy donor studied in parallel. Lower panel: densitometric analysis and statistical significance (\*\*p<0.01) on five patients and five NDs are shown. Values corresponding to unstimulated cells were subtracted.



### 3.5.3 Defective I $\kappa$ B $\alpha$ phosphorylation in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells

We then analyzed the activation of another family of transcription factor, NF $\kappa$ B, which is involved in the transcription of IL-2 and other cytokine genes. In resting cells NF $\kappa$ B proteins are retained in the cytoplasm through binding to I $\kappa$ B proteins. Upon receptor triggering, I $\kappa$ B proteins are rapidly phosphorylated and therefore targeted for ubiquitination and proteasome-mediated degradation. I $\kappa$ B degradation allows nuclear translocation of NF $\kappa$ B dimers. We analyzed the phosphorylation of I $\kappa$ B $\alpha$  induced by stimulation of patients and control CD4<sup>+</sup> T cells by TCR cross-linking with anti-CD3 plus anti-CD28 mAb. As shown in Fig.7, I $\kappa$ B $\alpha$  was phosphorylated in control CD4<sup>+</sup> T cells after 30 min of stimulation and still sustained after 2 hours. Conversely, in ADA<sup>-/-</sup> cells the levels of phosphorylated I $\kappa$ B $\alpha$  remained low or undetectable for the time points examined (Fig.7,  $p < 0.05$  vs ND). Remarkably, pre-transcriptional events involving I $\kappa$ B $\alpha$  occurred almost normally in gene-corrected cells, resulting in the correction of the cytokine transcriptional defect (Fig.7).



**Figure 7. Analysis of IκBα phosphorylation in CD4<sup>+</sup> T-cell lines from ADA-SCID patients.** Anti-phospho-IκBα of CD4<sup>+</sup> T cells stimulated as described above. Filters were stripped and re-probed with an anti-GAPDH mAb. Upper panel: immunoblots from two patients and two representative healthy controls are shown. Lower panel: cumulative data of densitometric analysis from four patients and four NDs are presented. Results of the statistical analysis are represented as follows: \* p<0.05; \*\* p<0.01. Values corresponding to unstimulated cells were subtracted.

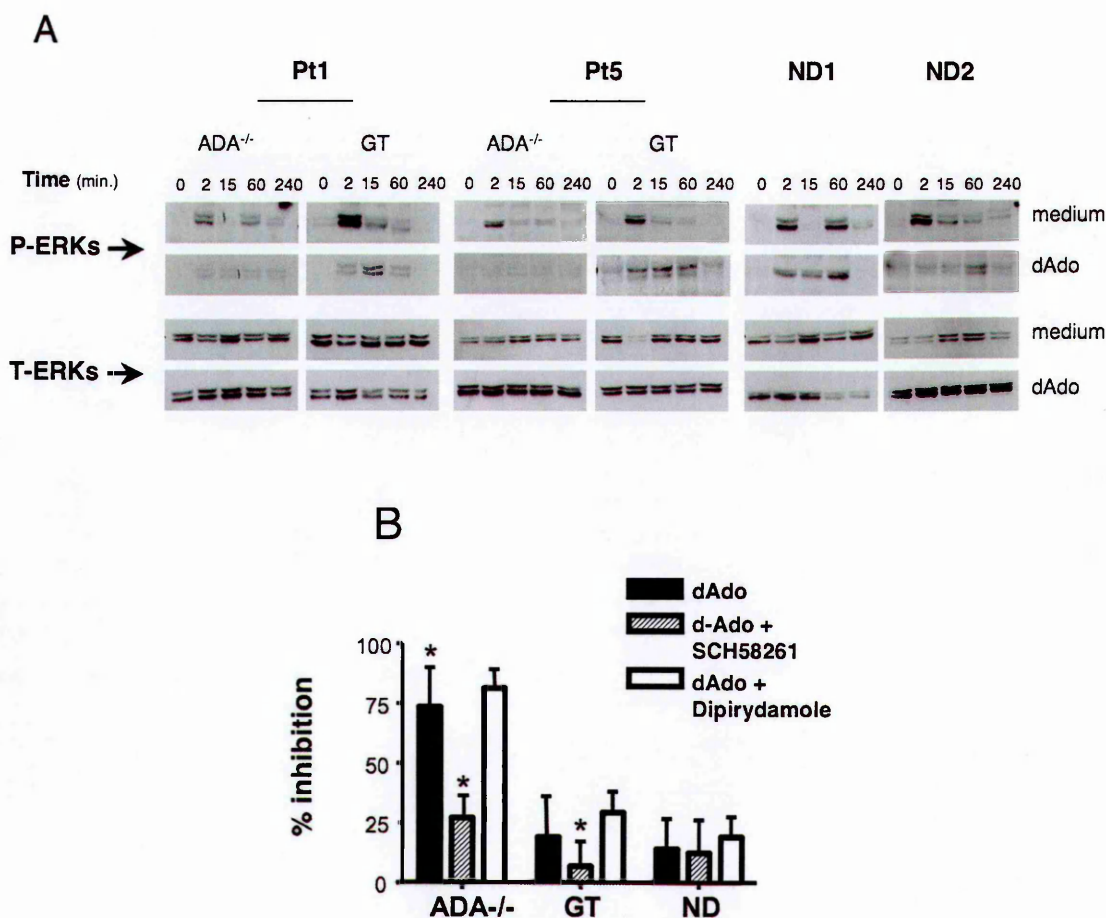
### 3.6 dAdo completely abrogates T-cell activation and function in ADA<sup>-/-</sup> T cells

In order to further dissect the biochemical events linking an altered purine metabolism to the immune defect, we exposed T cells from patients and controls to ADA-SCID-like *in vivo* conditions. The most striking metabolic alteration in ADA-SCID is the massive accumulation of dATP in erythroid cells (Hirschhorn, 1993) (Hershfield, 1998). Since dATP levels directly reflect the amount of dAdo excreted in the body fluids, this ADA metabolite may have more relevant clinical impact with respect to Ado. For these reasons, we studied T cell activation and effector functions in the presence of exogenously added dAdo at concentrations expected to be found in

ADA-SCID patients (500  $\mu\text{mol/L}$ ). We first investigated the activation of ERKs signaling.  $\text{CD4}^+$  T cells from ADA-SCID patients and healthy donors were stimulated with cross-linked anti-CD3 plus anti-CD28 mAbs in absence or presence of dAdo, and activation of ERK1/2 pathway was examined by western blot. Previous results have indicated that ERKs phosphorylation was consistently reduced in  $\text{ADA}^{-/-}$   $\text{CD4}^+$  T cells. Treatment with dAdo almost completely abolished ERKs activation in ADA-deficient cells, indicating a TCR-antagonizing effect (Fig. 8A).

Exposure to dAdo during TCR/CD28-mediated stimulation did not affect the induction of ERKs expression in GT (Pts  $n=3$ ) and control cells ( $n=3$ ) (Fig. 8A). However, it resulted in delayed kinetics of ERKs activation, as indicated by the shifted peaks of phosphorylation (Fig. 8A), thereby providing clear evidence for an existing cross-talk between TCR- and dAdo-triggered biochemical pathways. Accordingly, the same concentration of dAdo strongly inhibited T cell proliferation driven by optimal TCR stimulation (1  $\mu\text{g/ml}$  anti-CD3) of  $\text{ADA}^{-/-}$   $\text{CD4}^+$  cells ( $p<0.05$  vs NDs, Fig. 8B and data not shown). In contrast, GT cells were only minimally affected, not differently from healthy controls (Fig. 8B and data not shown). Since this concentration of dAdo did not affect cell viability (data not shown), these results prove that dAdo can inhibit TCR-triggered activation independently from a direct cytotoxic effect.

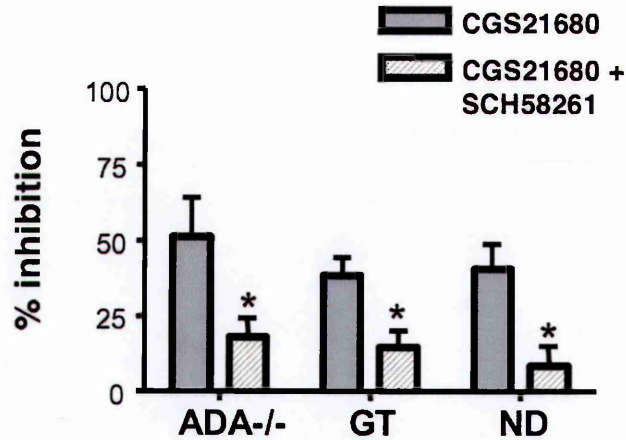
Figure 8



**Figure 8. dAdo strongly inhibits ERKs activation and effector functions in ADA-deficient T cells.** (A) ERKs activation was analyzed in CD4<sup>+</sup> cells with the stimulation protocol described above in absence or presence of dAdo (500  $\mu$ mol/L). The blots corresponding to basal (medium) P-ERKs and T-ERKs expression are the same depicted in Fig.5 for Pt1, Pt5 and ND1. Samples of untreated and dAdo-treated stimulated cells were prepared in parallel, run on different gels but probed with the different Abs at the same time. (B) CD4<sup>+</sup> cells were induced to proliferate with 1  $\mu$ g/ml coated anti-CD3 mAb in the absence or presence of dAdo (500  $\mu$ mol/L). The specified compounds (SCH58261 or Dipirydamole) were included into the incubation medium 1 hr before the addition of dAdo. After 48h cell proliferation was assessed by [3H]thymidine incorporation. The results from five patients and five controls were expressed as percentage of inhibition with respect to the stimulated untreated conditions. Statistical analysis (\*  $p < 0.05$ ) was performed on c.p.m. values.

### **3.7 dAdo-related inhibitory effects are mediated by the extracellular A<sub>2A</sub> adenosine receptor signaling**

To date, similar suppressive properties on T cell function have been consistently attributed to Ado, by virtue of its ability to physiologically signal through Gs-protein coupled purinergic receptors (Klinger et al., 2002). Among them, the A<sub>2A</sub> receptor is the major adenosine receptor subtype expressed in human lymphocytes (Koshiba et al., 1999) (Thiel et al., 2003). To establish a potential role of A<sub>2A</sub> receptor-mediated extracellular signaling in the inhibitory effects exerted by dAdo, we investigated T-cell proliferation in the presence of the A<sub>2A</sub> R selective antagonist SCH58261. Under these conditions, the suppressive activity of dAdo on proliferation of ADA<sup>-/-</sup> T cells was significantly inhibited ( $p < 0.05$ , Fig. 8B). Conversely, blocking of nucleoside uptake by a strong inhibitor of the nucleoside transport system, Dipyridamole, had no protective effect, and slightly enhanced the inhibitory properties of dAdo, in both patients and control cells (Fig. 8B). The effect of Dipyridamole may likely rely on an increased dAdo extracellular availability, thereby amplifying its TCR-antagonizing activity. Remarkably, the selective A<sub>2A</sub> R agonist, CGS21680 (Jarvis et al., 1989), mimicked the suppressive effect of dAdo on cell proliferation, which, in turn, was blocked by the receptor antagonist SCH58261 (Fig. 9), confirming that the dAdo-mediated effects are initiated by an extracellular adenosine receptor signaling rather than by its intracellular metabolism.



**Figure 9. The selective  $A_{2A}$  receptor agonist mimics dAdo-induced inhibitory effect.**  $CD4^+$  cells were induced to proliferate with 1  $\mu$ g/ml coated anti-CD3 mAb in the absence or presence of the selective  $A_{2A}$  R agonist CGS21680 (100  $\mu$ mol/L). The  $A_{2A}$  R selective antagonist (SCH58261) was included into the incubation medium 1 hr before the addition of dAdo. After 48h cell proliferation was assessed by [3H]thymidine incorporation. The results from five patients and five controls were expressed as percentage of inhibition with respect to the stimulated untreated conditions (\* $p < 0.05$ ).

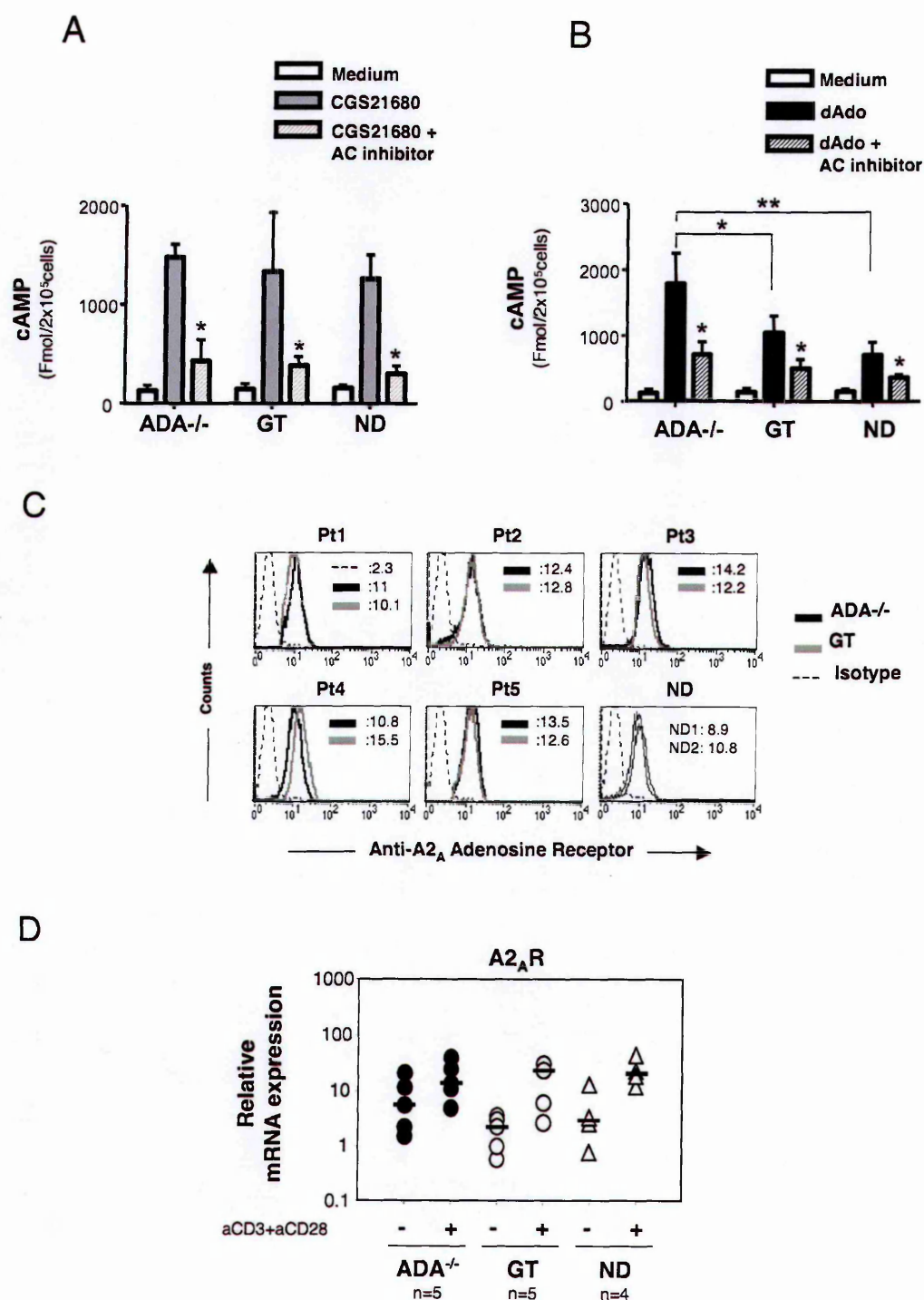
### 3.8 dAdo induces higher intracellular cAMP levels in $ADA^{-/-}$ $CD4^+$ T cells

The Gs-protein coupled  $A_{2A}$  adenosine receptor signal to increase intracellular cAMP (Klinger et al., 2002), which is a well-known negative regulator of T cell function (see section 1.9.2). Accordingly, exposure to selective  $A_{2A}$  receptor agonist (1 mmol/L) induced elevation of intracellular cAMP at similar extent in both control and patients' T cells (Fig. 10A). Measurement of cAMP levels in T cells cultured in presence of dAdo (5 mmol/L) showed a strong increase over the basal concentration in both ADA-SCID patients and healthy controls (Fig. 10B). However, the intracellular cAMP levels were 2-fold higher in  $ADA^{-/-}$  cells compared to normal and GT cells ( $p < 0.05$  vs ND and GT). To investigate whether the higher induction of intracellular cAMP in  $ADA^{-/-}$  cells could be attributed to difference in receptor density, we analyzed

the expression of A<sub>2A</sub> adenosine receptor in resting and activated state. Expression of A<sub>2A</sub> R in resting condition is similar in ADA-deficient, GT and healthy control cells, as assessed at protein level, by intracellular staining, or at mRNA level by RT-PCR (Fig. 10C-D). Upon TCR/CD28 stimulation the expression is markedly upregulated in ADA<sup>-/-</sup> cells as in control and GT cells (Fig10D). Collectively, these results indicate that the dAdo-triggered abrogation of T cell function in ADA<sup>-/-</sup> cells is mediated through A<sub>2A</sub> adenosine receptor and is cAMP-dependent, thus strengthening the proposed concept of ADA-SCID as a signaling disease.



Figure 10



**Figure 10. cAMP levels and Ado receptors expression in ADA-deficient cells.** (A) Intracellular cAMP levels were determined, as described in the methods, in CD4<sup>+</sup> T cells incubated with culture medium containing 1mmol/L CGS21680 or (B) 5 mmol/L dAdo for 15 minutes. The inhibitor of adenylyl cyclase (AC) (1mmol/L) was added 30 min before. The data represents the mean  $\pm$  SD from five patients and four NDs in one out of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ . (C) Expression of A<sub>2A</sub> adenosine receptor in gated CD4<sup>+</sup> T cells. Intracytoplasmic staining was performed in resting cells and M.F.I. values were indicated in the histogram plots. (D) Levels of A<sub>2A</sub> R mRNA in CD4<sup>+</sup> T cells from patients and healthy



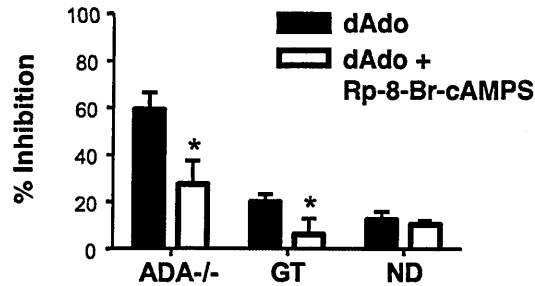
controls. Total RNA was isolated from either resting or stimulated (1 $\mu$ g/ml anti-CD3 plus 10  $\mu$ g/ml anti-CD28 mAbs) cells and adenosine receptor gene expression was measured by quantitative RT-PCR using the standard curve of a reference sample. Values were measured in duplicates, and normalized by the expression of the housekeeping gene HPRT.

### **3.9 PKA hyperactivation is the downstream effector of dAdo suppressive function**

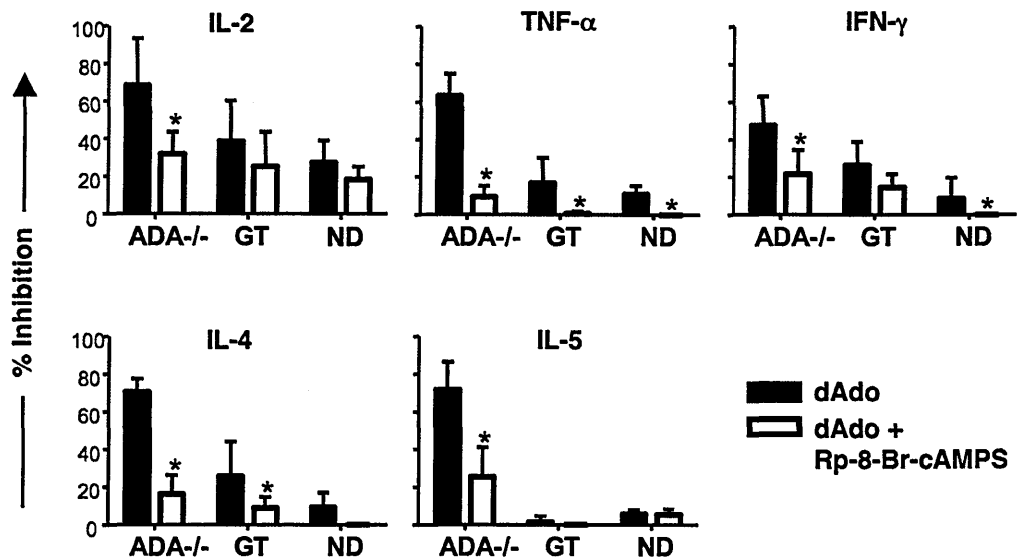
With few exceptions, the effects of cAMP are mediated by cAMP-dependent protein kinase A type I (PKAI) (Torgersen et al., 2002). This isoenzyme is thought to play a major role in cAMP-mediated effects in T lymphocytes, because it redistributes and colocalizes with the T-cell receptor in lipid rafts during T cell activation (Vang et al., 2001). In order to explore the potential involvement of PKA hyperactivation in the dAdo-mediated suppressive activity, we used a specific membrane-permeable inhibitor of cAMP-dependent protein kinases I and II activation, Rp-8-Br-cAMPS (Gjertsen et al., 1995). Addition of PKA inhibitor significantly reduced the inhibitory effect exhibited by dAdo on proliferative responses of ADA<sup>-/-</sup> cells (Fig. 11A;  $p < 0.05$ ). No significant improvement in anti-CD3-induced proliferation was observed in ADA<sup>-/-</sup> cells when Rp-8-Br-cAMPS alone was added to cell culture (data not shown), suggesting that the intrinsic defect is not related to chronically elevated endogenous cAMP levels. Exposure to dAdo had a broad suppressive effect even on cytokine production in T cells, with the most important alterations in ADA<sup>-/-</sup> cells (Fig. 11B and data not shown). Therefore, we examined the effect of Rp-8-Br-cAMPS on cytokine production ability of anti-CD3-stimulated CD4<sup>+</sup> T cells from five ADA-SCID patients and five controls. In presence of PKA antagonist, the suppressive activity of dAdo on cytokine production ability of ADA<sup>-/-</sup> T cells was significantly inhibited (Fig. 11B). These results indicate that dAdo inhibits T cell activation in ADA-SCID patients through a cAMP/PKA-dependent pathway.

Figure 11

A



B



**Figure 11. PKA hyperactivation is responsible for dAdo-mediated suppression of T cell function in ADA-deficient cells.** CD4<sup>+</sup> T cells were stimulated with 1μg/ml coated anti-CD3 mAb in the presence or absence of dAdo (500 μmol/L) with or without the inhibitor of cAMP-dependent protein kinase A, Rp-8-Br-cAMPS (150 μmol/L). Rate of proliferation was evaluated by [3H]thymidine incorporation after 48 hours (A) while supernatants were collected for cytokine production quantification by ELISA after 18 (IL-2) or 48 hours (all other cytokines) (B). The percentages of inhibition of proliferation and cytokine production in the presence of the drugs relative to control are indicated. The data represents the mean ± SD of five patients and five healthy controls from one of three independent experiments. Statistical analysis (\*p<0.05) was performed on real values.

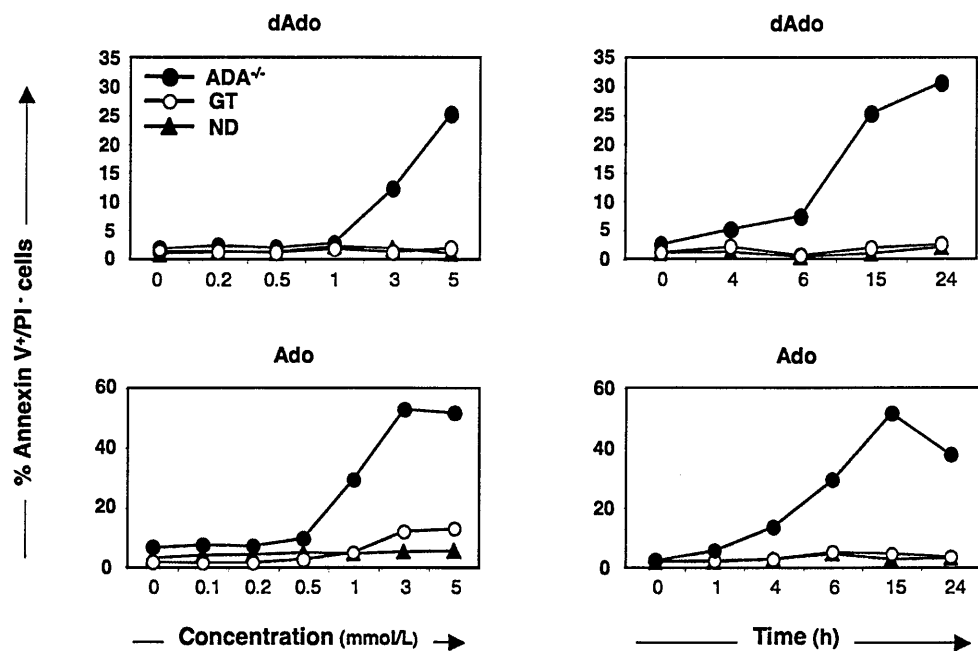
### **3.10 Increased susceptibility to apoptosis in ADA<sup>-/-</sup> cells upon exposure to ADA substrates**

The metabolic consequences associated with ADA deficiency have been demonstrated to trigger apoptosis in cultured mouse thymocytes (Van De Wiele et al., 2002) and peripheral T cells treated with an ADA inhibitor (Kizaki et al., 1990) (Benveniste and Cohen, 1995). To determine whether apoptosis may play a major role in the T lymphocytopenia observed in ADA-SCID patients, T cells were exposed to increasing concentrations of dAdo or Ado for different time, and apoptosis was assessed by Annexin V/PI staining. A different kinetic in terms of time and concentration dependence was showed for both ADA substrates, with Ado displaying a stronger apoptosis-inducing effect than dAdo. Indeed, longer treatments (15 hours vs 6 hours) and relatively higher concentrations (5 mmol/L vs 1mmol/L) were required by dAdo to induce a comparable percentage of cell death in ADA<sup>-/-</sup> cells (Fig. 12A). Cumulative results from five patients confirmed that treatments with ADA substrates induced a three- to four-fold higher proportion of apoptotic cells in ADA<sup>-/-</sup> T cells, as compared to healthy control cells. In contrast, GT cells were rescued from apoptosis as control cells, in agreement with the expression of functional ADA (Fig. 12B). To better understand the metabolic pathway involved, we evaluated the effects dAdo/Ado on cell viability in the presence of nucleoside transporter inhibitors. As shown in Figure 12B, Dipyridamole was able to completely prevent Ado- but not dAdo-mediated apoptosis in ADA-deficient cells. Similar effect was obtained using an additional inhibitor of the nucleoside transport, NBTI (data not shown). These data suggest that Ado-induced cell death is due to its direct intracellular action consequent to a membrane entry through the equilibrative nucleoside transporter. Since neither Dipyridamole nor NBTI (data not

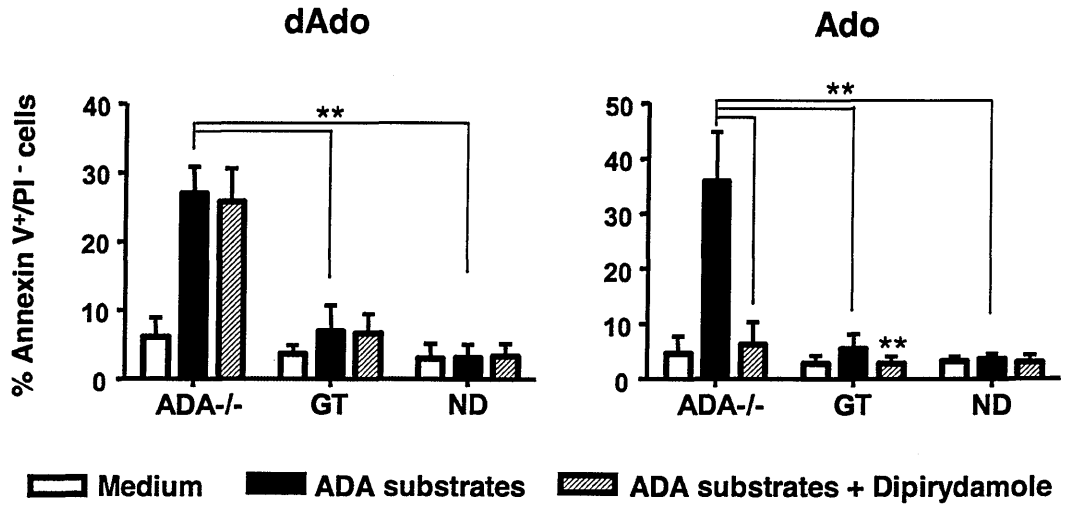
shown) failed to protect ADA<sup>-/-</sup> cells from dAdo-triggered apoptosis, we explored whether it was acting at a cell-surface site through an extracellular signaling involving A<sub>2A</sub> Adenosine receptors. No rescue from apoptosis was observed in ADA-deficient cells when exposed to dAdo in presence of non per-se toxic doses of the A<sub>2A</sub> Adenosine receptor selective antagonist SCH58261.

Figure 12

A



B



**Figure 12. Dose- and time-dependent apoptosis induced in ADA-deficient T cells by ADA substrates.** (A) Bulk T cell-lines were incubated with the indicated concentrations of purine metabolites and cultured for 15h (dAdo) or 6h (Ado). In time course experiments, T cells were incubated with dAdo (5mmol/L) or Ado (1mmol/L) for the indicated period. The percentages of early apoptotic cells (Annexin V<sup>+</sup>/PI<sup>-</sup>) are shown for Pt3 and one healthy control as representative experiment. (B) Bulk T cell-lines from five ADA SCID patients and six healthy controls were cultured for 15h with dAdo (5mmol/L) or 6h with Ado (1mmol/L) in the absence or the presence of the inhibitor of nucleoside transporter, Dypiridamole (1μmol/L). The percentages of Annexin V<sup>+</sup>/PI<sup>-</sup> cells as mean  $\pm$  SD are shown. Data are representative of at least 5 independent experiments. (\*\*p<0.01).

Altogether, these studies on the molecular mechanisms involved in ADA-deficient T cells unresponsiveness to TCR/CD28-mediated stimulation, indicate that ADA-deficient environment affect the ability to transduce activating signals through the T-cell receptor, and support the role of aberrant extracellular signaling in the disease pathogenesis.

### **3.11 Analysis of retroviral vector integrations in hematopoietic cells from ADA-SCID patients: *In vitro* vs *Ex vivo* integrations**

We have performed a systematic analysis to identify and map vector insertion sites in ADA-SCID patients treated with CD34<sup>+</sup> cell gene therapy. These studies are aimed at defining the pattern of retroviral integrations, their potential influence on gene expression and the analysis of the clonal composition of transduced HSC and their progeny. The genome-wide analysis of RIS was carried out on BM and peripheral blood (PB) cell populations derived from five ADA-SCID patients (Pt1-5) before and 1.5 to 47 months after GT. Vector-genome junctions were cloned from pre-transplant CD34<sup>+</sup> cells (*in vitro*) or post-GT hematopoietic cells (*ex vivo*) by inverse or linker-mediated (LM) PCR, sequenced and mapped onto the human genome. Overall, 212 *in vitro* and 496 *ex vivo* integrations could be unambiguously assigned to a unique chromosomal position.

#### **3.11.1 Genomic distribution of RV integration sites in ADA-SCID gene therapy**

Recent studies have indicated that gammaretroviral vectors integrate in a non-random fashion in their host genome, but information on the distribution of retroviral insertion sites (RIS) in human long-term reconstituting HSC following therapeutic gene transfer is still limited. The distribution of RIS with respect to RefSeq genes is shown in Table 3. Integrations within the transcribed portion of genes were more represented in the *in vitro* than in the *ex vivo* sample (50.9 vs 41.3%,  $p=0.03$ ). Although both values were significantly higher than the frequency of annotated genes in the human genome (35.5%,  $p<0.01$ ), the proportion of intragenic hits *ex vivo* was not significantly different from that obtained from a collection of 398 random sequences from a control DNA library (37.9%,  $p=0.3$ ). Exons were rarely hit, both *ex vivo* (2.0%) and *in vitro* (0.5%), in agreement with the overall occupancy of coding sequences in the human genome

(1.8%). We then examined the distribution of vector integrating in intergenic regions, and determined the distance to the nearest 5' and 3' ends of genes flanking each RIS. Integrations <30 kb upstream of a gene were over-represented with respect to those <30 Kb downstream, particularly in the *ex vivo* sample, and were more frequent *ex vivo* than *in vitro* in the 10 kb region upstream from the TSS (Table 3). As reported for human cell lines and for hematopoietic cells in primates (Wu et al., 2003) (Hematti et al., 2004), integrations within a 5-kb window on either side of the nearest TSS were also over-represented in both samples (23.6 and 28.8%) (Table 3 and Fig. 13A). A strong preference for gene-dense chromosomal regions was observed in the CD34<sup>+</sup> cell pre-transplant sample, with 66.1% of RIS landing in genomic regions containing >10 genes per Mb, as compared to the 17% expected from the density of RefSeq genes in the human genome (Fig. 13B). This bias was maintained in the HSC progeny post-transplant (70.6%), with a further enrichment in regions containing >20 genes per Mb (Fig. 13B). Accordingly, integrations retrieved from both samples were unevenly distributed in human chromosomes, with a tendency to correlate with gene density rather than chromosome size (Fig. 13C). Clusters of insertions were observed on chromosome 11p13 (8 sites), 18q21.33, 19p13.3 (7 sites), 1q32.1, 10p15.1, 12p13.32, 17q12, 17q23.2 (6 sites), and 1q21.2, 6q25.3, 11p11.2, 15q26.1, 17p13.1 (5 sites).

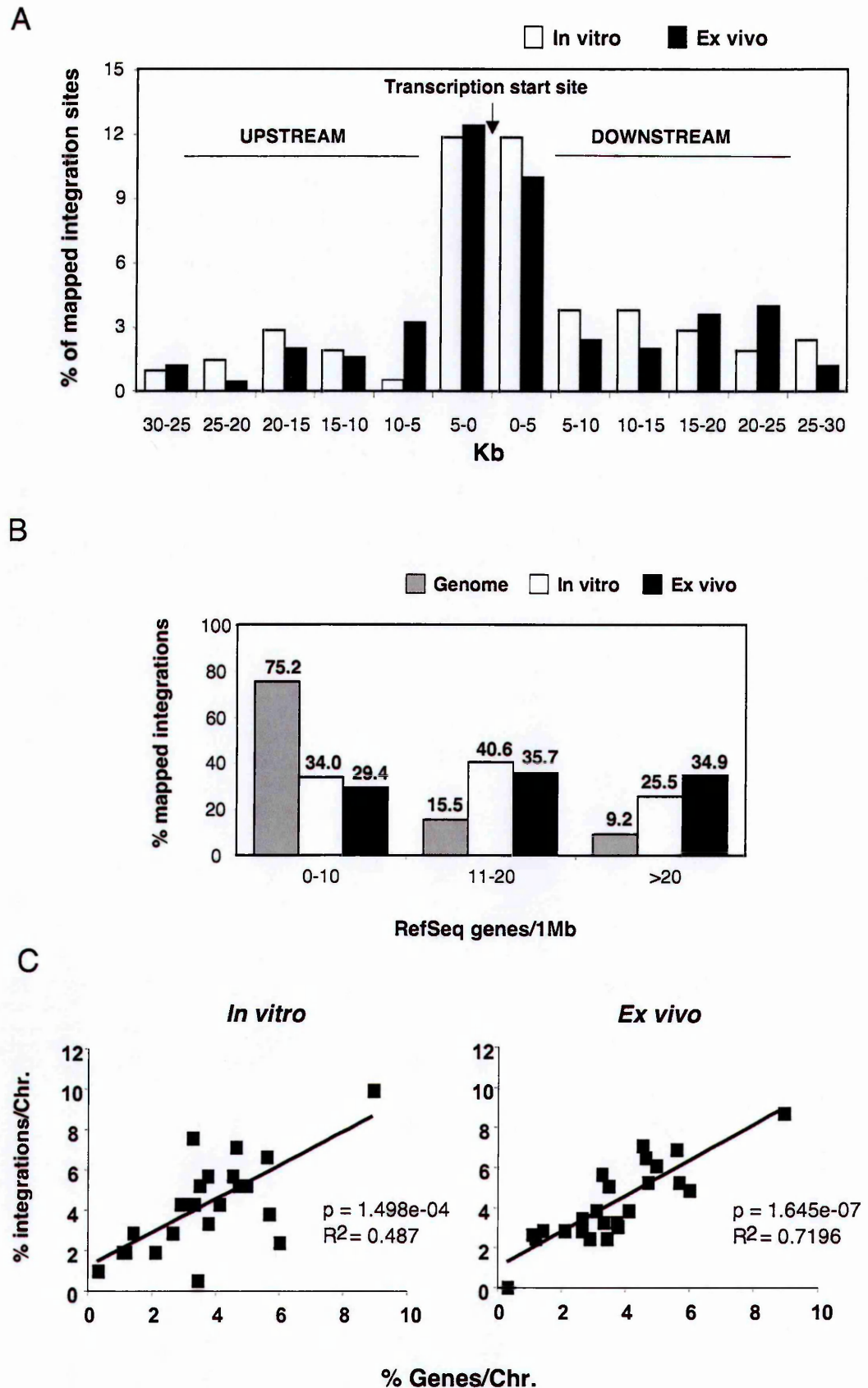
	Intragenic (%)	Intergenic (%)	<30 kb upstream (%)	<10 kb upstream (%)	<30 kb downstream (%)	±5 kb from TSS (%)
<b>In vitro (pre-transplant) CD34<sup>+</sup> cells (212 hits)</b>	<b>50.9<sup>*</sup></b>	<b>49.1</b>	<b>19.4</b>	<b>12.3<sup>**</sup></b>	<b>4.3<sup>°</sup></b>	<b>23.6<sup>**</sup></b>
<b>Ex vivo follow up (496 hits)</b>	<b>41.3<sup>^</sup></b>	<b>58.7</b>	<b>25.6<sup>**</sup></b>	<b>19.6<sup>**§</sup></b>	<b>12.7</b>	<b>28.8<sup>**</sup></b>
<b>Total (708 hits)</b>	<b>44.3</b>	<b>55.7</b>	<b>23.7</b>	<b>17.4</b>	<b>10.2</b>	<b>27.3</b>
<b>Expected in the human genome</b>	<b>35.5</b>	<b>64.5</b>	<b>n.d.</b>	<b>n.d.</b>	<b>n.d.</b>	<b>n.d.</b>
<b>Expected from random library</b>	<b>37.9</b>	<b>62.1</b>	<b>14.5</b>	<b>4.2</b>	<b>10.5</b>	<b>6.1</b>

**Table 3. Retroviral integration site distribution in hematopoietic cells from ADA-SCID patients.** Distribution of retroviral integration sites unambiguously mapped in CD34<sup>+</sup> HSC from four ADA-SCID patients before transplantation (*in vitro*, pre-transplant CD34<sup>+</sup> cells, 212 hits), from BM- or PB-derived hematopoietic cells from five ADA-SCID patients 1.5 to 47 months after gene therapy (496 hits), or total *in vitro* and *ex vivo* integrations (708 hits). Integrations were distributed as inside (intragenic) or outside (intergenic) RefSeq genes, at a distance of <30 kb or <10 kb upstream or <30 kb downstream from genes, and at a distance of ±5 kb from transcription start sites (TSS). <sup>1</sup>Values expected for the human genome (NCBI35 annotation). <sup>2</sup>Values obtained from a collection of 398 sequences randomly cloned from a library of *Pst*I/*Mse*I-restricted, LM-PCR-amplified human DNA.

<sup>\*</sup>p<0.00001 vs human genome, p<0.01 vs random, p<0.05 vs *ex vivo*; <sup>^</sup>p<0.01 vs human genome; <sup>\*\*</sup>p<0.001 vs random; <sup>§</sup>p<0.05 vs *in vitro*; <sup>°</sup>p<0.05 vs random. p<0.001 vs *ex vivo*.



Figure 13



**Figure 13. Genomic distribution of RV integration site in ADA-SCID patients.** (A) Distribution of integration sites within 30 kb window around the transcription start site. Each bar corresponds to the frequency of integration sites retrieved by LM-PCR technique in pre-

transplant CD34<sup>+</sup> cells (*in vitro*, n=212) or post-GT cells (*ex vivo*, n=251) within a 5-Kb interval from the transcription start site of the nearest gene. (B) Correlation between integration sites and gene density within a 1Mb window. The number of RefSeq genes was determined in a region of 500 kb on either side of integration site for both “*in vitro*” and “*ex vivo*” samples. The data were compared to *in silico*-generated random integration sites (Hematti et al., 2004). (C) Correlation between the percentage of integration events and the percentage of genes on individual chromosomes. The number of genes per chromosome refers to NCBI version 35 of human genome. R<sup>2</sup> Goodness of fit; p value was determined using the F statistic.

### 3.12 Retroviral integration hot spots in ADA-SCID gene therapy

We then asked the existence of preferred regions of RV integration. We identified “hot spots” by the occurrence of 2 independent integrations landed within the transcribed portion of a RefSeq gene or  $\geq 2$  independent integrations within arbitrarily chosen windows of 30 kb (2 hits), 50 kb (3 hits) or 100 kb ( $\geq 4$  hits) (Suzuki et al., 2002). Cumulatively, 50 genomic loci were recurrently targeted by RV integration, of which 21 were in common between the *in vitro* and *ex vivo* sample (Table 4A-B). Overall, 14.6% (31/212) of *in vitro* integrations and 15.9% (79/496) of *ex vivo* integrations were classified as “hot spots” (Table 4A-B). The most frequently hit genes ( $\geq 3$  times) encoded a protein kinase (*DYRK1A*), a putative RNA-binding protein (*RNPC1*), and a cell cycle regulator (*CCND2*), or were involved in tumor-associated chromosomal translocations (*BCL2*, *BLM*, *LMO2*) (Table 4B and Fig. 14). The *MDS1/EVII* locus, which has been identified at relatively high frequency in previous mapping studies of retroviral integration sites (Wu et al., 2006) (Calmels et al., 2005) (Ott et al., 2006a), was hit only once, in granulocytes from Pt3 6 months post-GT and became undetectable at later time points by sequence-specific PCR (data not shown). *LMO2* was overall the most frequently hit locus, with one integration cloned from pre-transplant CD34<sup>+</sup> cells (Pt5), two from granulocytes (Pt5) and three from T cells (Pt1, Pt3, Pt4) (Fig. 14). Five integrations were located outside the gene, of which three within 39 bp in a region at 39.2 Kb from the start site (S3\_042: Pt3 18 months post-GT;

S5\_144: Pt5, 6 mo post-GT; S5\_P048: Pt5 pre-infusion). The other integrations were located 15 Kb upstream (S4\_048: Pt4, 26 mo post-GT), 1.3 Kb upstream (S1\_049: Pt1, 27 mo post-GT) and inside the second intron (S5\_163: Pt5, +12 mo post-GT) (Fig.14). All integrations were detected occasionally in one sample, and represented only a small proportion of the sequences isolated by shotgun cloning.

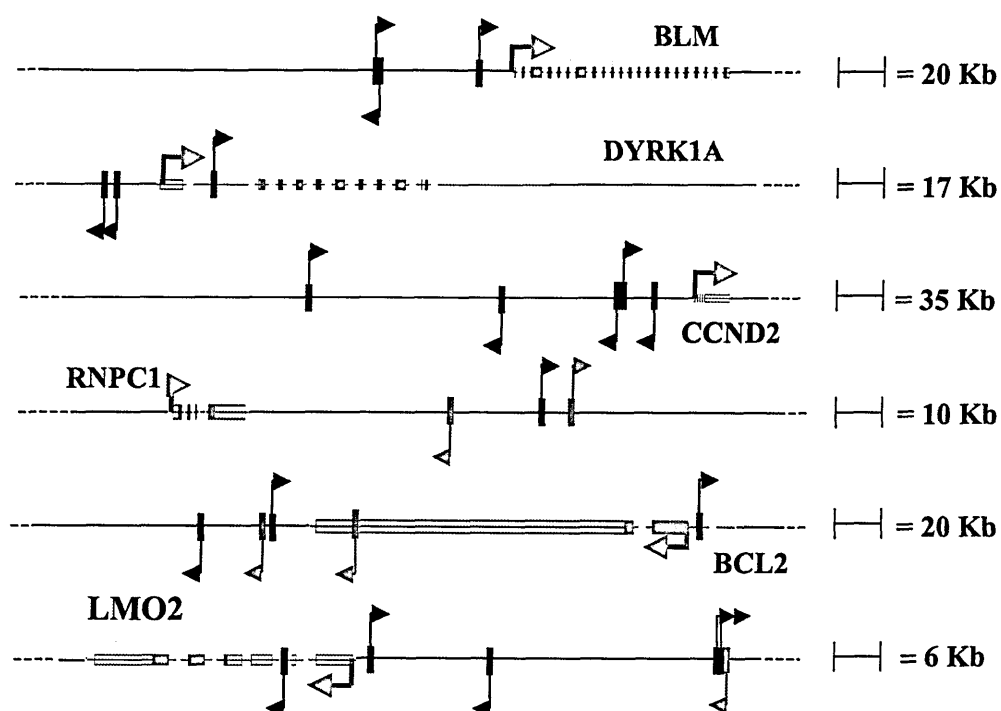
<b>Integration n°</b>	<b>Chr. n°</b>	<b>Relative distance (bp)</b>	<b>Position</b>	<b>RefSeq gene</b>
S3_036 S3_P029	10	4874	Inside	<b>ADD3</b>
S5_042 S3_P091	3	20281	Inside	<b>BBX</b>
S3_086 S3_P007	19	17343	Inside	<b>Q8IUV1_HUMAN</b>
S5_176 S3_P049	5	4000	Inside	<b>Q8N8D9_HUMAN</b>
S5_010 S5_P002	22	918	Inside	<b>TNRC6B</b>
S3_058 S3_P054	8	140308	Inside	<b>TRPS1</b>
S1_062 S3_P024	3	34426	Inside	<b>NP_002213.1</b>
S5_049 S5_P020	9	44506	Inside	<b>CDW92</b>
S3_135 S3_053	1	17329	Inside	<b>PDE4B</b>
S2_031 S5_006	9	1353	Inside	<b>MLLT3</b>
S3_054 S5_022	16	12790	Inside	<b>BANP</b>
S1_114 S5_151	22	760	Inside	<b>NP_001008496.1</b>
S2_022 S4_041	22	4130	Inside	<b>YVO3_human</b>
S5_045 S5_173	6	66604	Inside	<b>Q8TEE4_human</b>
S3_063 S4_030	8	322230	Inside	<b>Q96Q05_human</b>
S4_P004 S5_P039	10	344	Inside	<b>C10orf107</b>
S3_P043 S5_P047	9	1811	Inside	<b>SHB</b>
S2_P054 S4_P027	10	87528	Inside	<b>ABLIM1</b>

**Table 4A. Hotspots: list of genes hit independently by two integration sites.** The relative distance refers to the distance between the two insertions inside the target gene. All insertions were <30 kb apart, with the exception of *ABLIM1*, *TRPS1*, *NP\_002213.1*, *Q8TEE4\_human*, *CDW92* and *Q96Q05\_human*. Three of these double hits were present in the pre-transplant sample, 7 in the post-transplant sample, and 8 in both.

Integration n°	Chr. n°	Relative distance (bp)	Position	RefSeq gene
S2_033 S4_017	10	25789	Upstream Upstream	<b>AKR1CL2</b>
S5_114 S2_P008 S5_076 S2_P015	18	73653	Downstream Downstream Downstream Inside	<b>BCL2</b>
S1_092 S3_108 S2_036	15	41359	Upstream Upstream Upstream	<b>BLM</b>
S2_045 S5_026	7	27387	Upstream Inside	<b>CCM2</b>
S5_138 S5_034 S1_021	12	29994	Upstream Upstream Upstream	<b>CCND2</b>
S5_071 S3_P018	2	1846	Upstream Upstream	<b>CYP1B1</b>
S3_046 S4_016 S3_111	21	14290	Upstream Upstream Inside	<b>DYRK1A</b>
S2_004 S2_019	X	297	Downstream Downstream	<b>EIF2S3</b>
S1_042 S3_P047	9	7309	Upstream Upstream	<b>GADD45G</b>
S3_P097 S5_033	6	1178	Upstream Upstream	<b>BTN3A2</b>
S5_056 S5_086	21	28407	Upstream Upstream	<b>HRMT1L1</b>
S5_P026 S3_P072	5	99	Upstream Upstream	<b>HTR4</b>
S1_023 S4_012	1	1879	Upstream Upstream	<b>IRF2BP2</b>
S3_109 S5_040	13	243	Upstream Upstream	<b>LCP1</b>
S5_163 S1_049 S4_048 S5_144 S3_042 S5_P048	11	58770	Inside Upstream Upstream Upstream Upstream Upstream	<b>LMO2</b>

S3_009 S5_014	8	642	Upstream Upstream	MTSS1
S1_011 S1_109	6	975	Upstream Upstream	MYCT1
S1_111 S3_130	14	6548	Downstream Downstream	NFKBIA
S2_007 S5_145	1	2445	Inside Upstream	NP_859071.1
S2_030 S2_032	2	1494	Upstream Upstream	PTMA
S5_136 S5_032	6	4123	Downstream Downstream	Q8TEE4_Human
S3_029 S3_081	9	4	Downstream Downstream	RANBP6
S5_093 S4_P033 S5_P051	20	28106	Downstream Downstream Downstream	RNPC1
S3_047 S4_047	3	4502	Downstream Downstream	RYBP
S3_P022 S4_050	21	52	Upstream Upstream	SAMSN1
S3_083 S5_P058	7	50	Downstream Downstream	SYPL
S2_021 S2_P033	17	2560	Upstream Upstream	UBP32_Human
S5_174 S4_P031	13	10670	Upstream Upstream	WBP4
S1_014 S1_036	20	15	Upstream Upstream	ZNF217
S5_157 S5_P028	8	3164	Upstream Inside	ZNF406
S2_P035 S5_092	1	180	Upstream Upstream	GMEB1
S2_P045 S5_072	21	685	Downstream Downstream	MRPL39

**Table 4B. Hotspots: List of genomic sites frequently hit by retroviral integrations.** The data report insertions from *in vitro* or *ex vivo* samples according to the following categories: 2 RIS within 30 Kb, 3 RIS within 50Kb,  $\geq 4$  RIS within 100Kb (Suzuki et al., 2002). In case of three or more integrations the relative distance refers to the external boundaries.

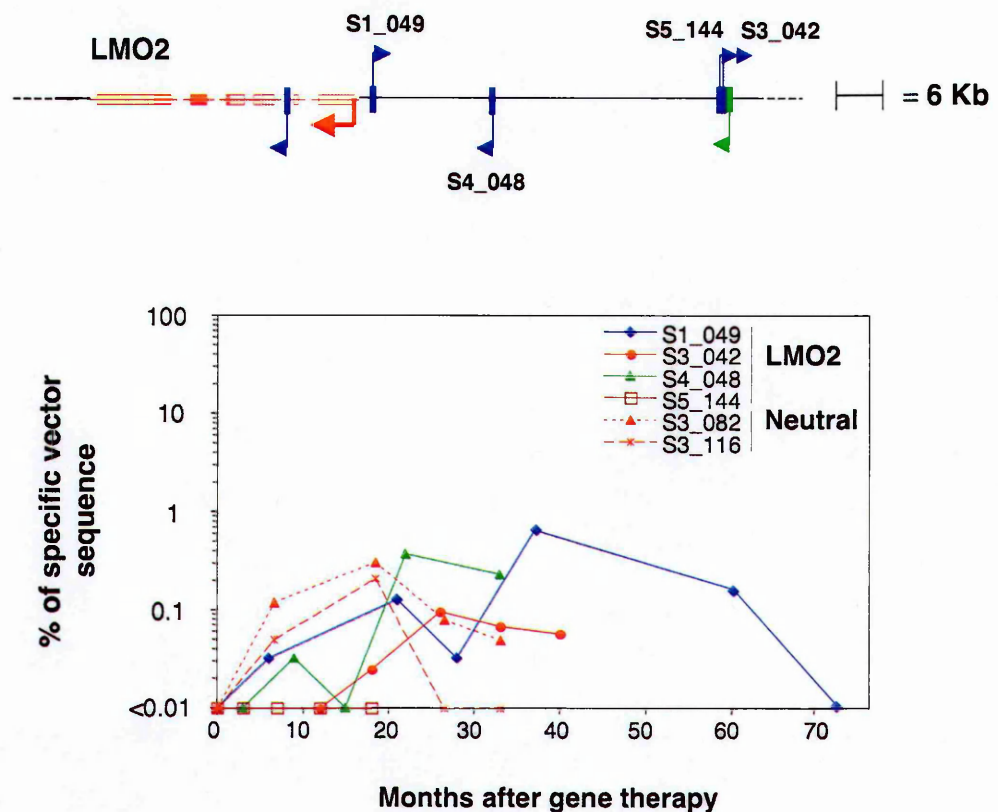


**Figure 14. Schematic representation of the most frequent hotspots found in ADA-SCID.** Integrated provirus is shown as blue (*ex vivo* integration) or green (*in vitro* integration) arrows. The genes are depicted in red with full boxes and arrows indicating the exons and the transcription start sites.

### 3.13 Long-term monitoring of *LMO2*-carryng clones

To assess the relative contribution of clones carrying *LMO2* insertions, we measured the frequency of four RIS by sequence-specific real-time PCR. Three integrations were detected in T cells from Pt1 (S1\_049), Pt3 (S3\_049) and Pt4 (S4\_048) from 6 up to 60 months post-GT, at levels fluctuating between 0.03% and 0.7% of the total CD3<sup>+</sup> populations (Fig. 15). Of notice, integration S1\_049 became undetectable at latest time of observation (6 years after GT). These integrations were not detectable in peripheral blood or BM purified granulocytes, BM erythroid cells and BM CD34<sup>+</sup> cells at repeated follow up controls, with the exception of CD34<sup>+</sup> cells from Pt1, which were positive 11 months after GT (0.15%) (data not shown). However, these frequencies are comparable to those observed for two non-recurrent insertions from Pt3 landed in

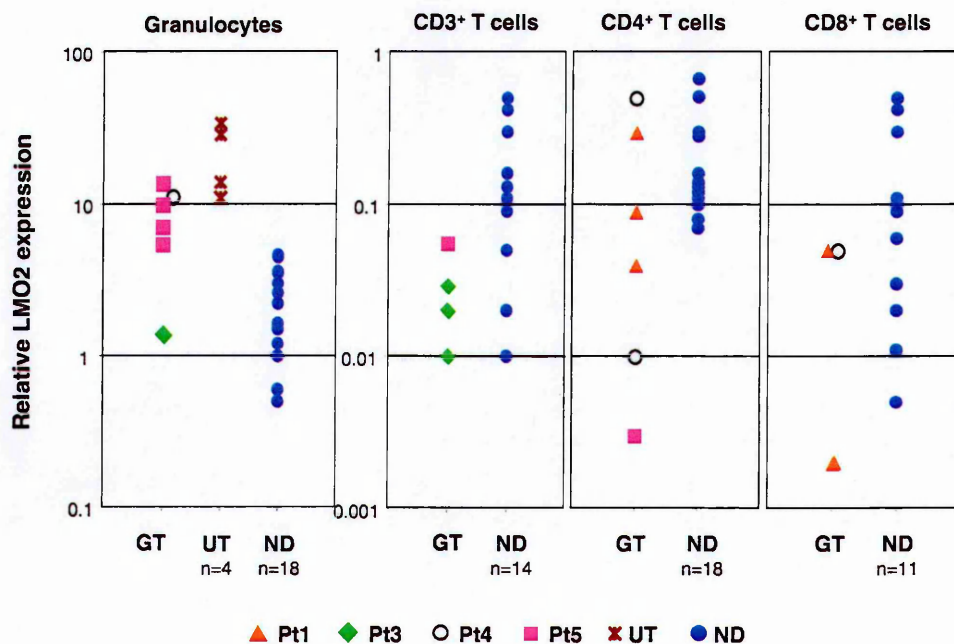
“neutral” region of the genome (Fig. 15). One integration (S5\_144) was undetectable in T cells and at the limit of PCR sensitivity in granulocytes (0.01%) from Pt5 (Fig. 15) but was absent in CD34<sup>+</sup> cells. B cells were weakly positive at occasional time points in Pt3, Pt4 and Pt5 (data not shown). These results indicated that clones carrying *LMO2* insertions undergo *in vivo* to normal physiological clonal fluctuations.



**Figure 15. Quantification of the contribution of the *LMO2* clones to the total T cell population.** The frequency of four insertions within the *LMO2* locus was measured by sequence-specific Real Time PCR in purified CD3<sup>+</sup> T cells isolated at different time points after gene therapy. In dotted lines are reported the frequencies of non *LMO2*-related (neutral) integrations retrieved from two CD4<sup>+</sup> T cell-clones of Pt3, as control of physiologic clonal fluctuations. The results are expressed as percentage of total CD3 population.

### 3.14 *LMO2* integrations do not lead to over-expression *in vivo*

To assess the potential influence of the nearby vector integration on *LMO2* gene expression, we measured mRNA levels in purified cell subpopulations from both untreated and treated ADA-SCID patients, and from healthy controls. No up-regulation of *LMO2* expression was observed in purified bulk T-cell subsets from GT treated patients compared to healthy controls (Fig. 16). *LMO2* expression in granulocytes was higher in untreated ADA-SCID patients than in healthy controls, and did not increase in samples from GT treated patients.

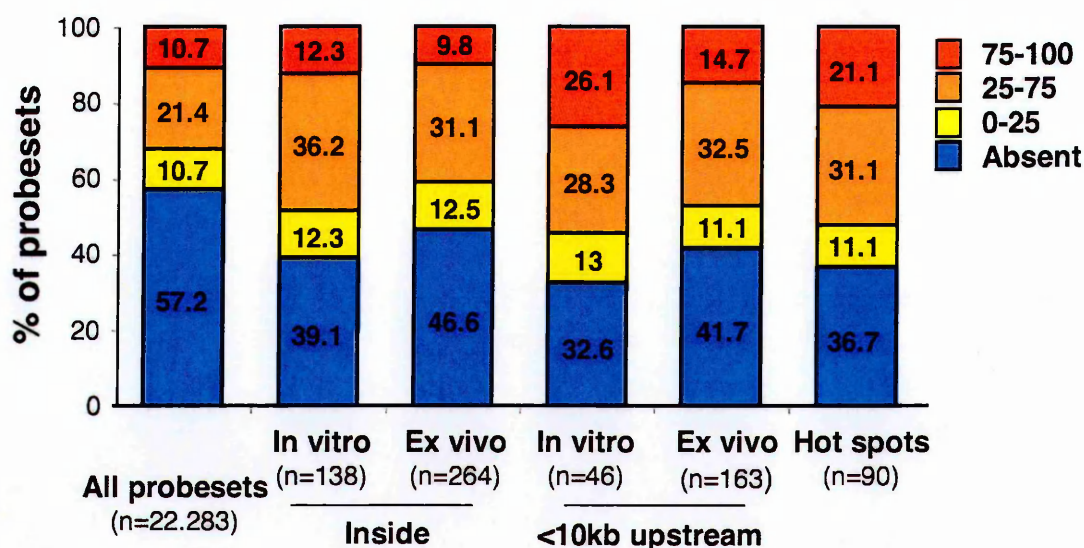


**Figure 16. Expression of *LMO2* in peripheral blood T cells and granulocytes.** Total RNA was isolated from freshly purified CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T cells or CD15<sup>+</sup> granulocytes of gene therapy-treated patients, ADA-SCID patients under PEG-ADA treatment (not treated with GT), and age-matched healthy controls. *LMO2* gene expression was measured by quantitative RT-PCR using the standard curve of a reference sample. Values were normalized on expression of the housekeeping gene HPTR and correspond to duplicate wells.



### 3.15 Integrations are found preferentially in/near genes expressed in CD34<sup>+</sup> cells

We next analyzed if the bias for the integration pattern near genes and specific hot spots reflected a preference for genes expressed in CD34<sup>+</sup> cells. To mimic the transcriptional activity at the time of gene transfer, we analyzed, by Affymetrix GeneChip® microarray analysis, the gene expression profile of cytokine-exposed BM CD34<sup>+</sup> cells under identical conditions required for the transduction procedure. The genes expressed and classified as “present” by the software were divided in 3 arbitrary categories comprising 0-25%, 25-75% and 75-100% of expression levels of all genes in a normalized distribution. The proportion of expressed genes in the array (42.8%) was significantly increased when the analysis was restricted to genes hit by integrations inside (60.9%) or within 10 Kb from TSSs (67.4%) in pre-transplant CD34<sup>+</sup> cells ( $p < 0.005$ , Fig. 17). Integrations upstream TSSs favored highly expressed genes, indicating that transcriptionally active regions are preferentially targeted by retroviral vectors in CD34<sup>+</sup> cells. Of interest, “hot spot” genes were more frequently expressed compared to the whole chip distribution (63.3 vs 42.8%), with a strong over-representation of highly expressed genes (21.1 vs 10.7%,  $p < 0.005$ ; Fig. 17). These data indicate that gene expressed in CD34<sup>+</sup> cells at the time of transduction were preferentially target by retroviral vector and may at least in part explain the preference for insertions retrieved in hot spots, including *LMO2*. The same tendency was observed for post-GT samples (*ex vivo*), although the frequency of “present” or highly expressed genes was lower compared to the pre-transplant profile (Fig. 17), suggesting a negative selection against integrations into active genes in hematopoietic cells *in vivo*.

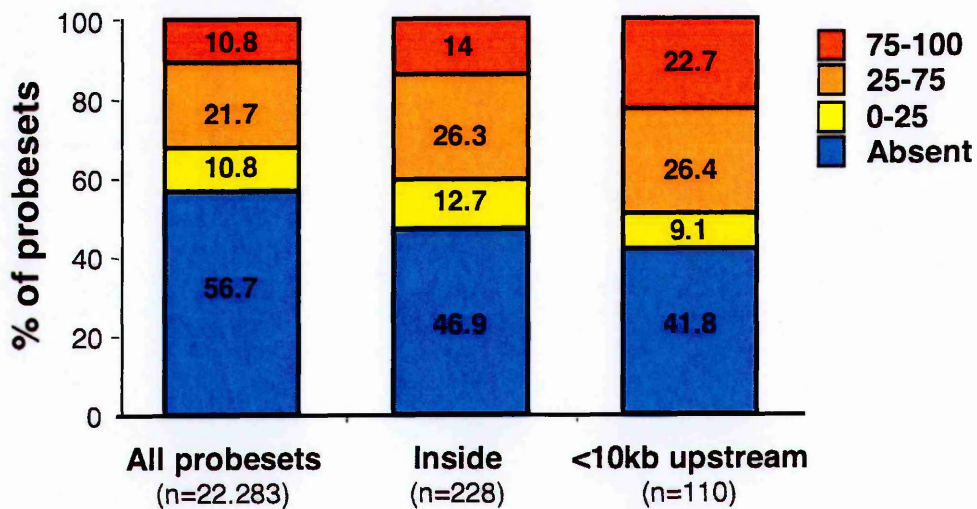


**Figure 17. Correlation between insertion sites and gene expression profile of CD34+ cells at the time of transduction.** The expression level of all probesets present on an Affymetrix HG-U133A microarray was compared to the expression level of probesets of genes targeted (inside or <10 kb upstream) or frequently hit (hot spots) by retroviral vector insertion in pre-infusion CD34+ cells (*in vitro*) and post-GT cells (*ex vivo*) or in both samples, respectively. The bars show the percentage distribution of expression levels classified as present and absent by MAS 5.0 software. The present probesets are then categorized according to two cut offs identified by first (0-25) and third (75-100) quartiles.

### 3.16 ADA selective advantage favors survival of vector clones integrating in/near genes transcriptionally active in T cells

Since T cells in ADA-SCID patients carry a selective advantage if ADA is expressed, we investigated whether the state of expression of a gene hit by an integration could influence the *in vivo* behavior of transduced T cells. The gene expression profile of purified T cells was determined and divided in arbitrary expression categories as described above (Fig. 18). Hit genes were expressed at a significantly higher frequency (53.1 vs 43.3%,  $p < 0.01$ ) and enriched in the high expression category (intragenic hits: 14.0 vs 10.8%,  $p < 0.05$ ; hits <10kb from TSS: 22.7 vs 10.8%,  $p < 0.005$ ). These data suggest that the selective pressure for vector-derived ADA expression favors

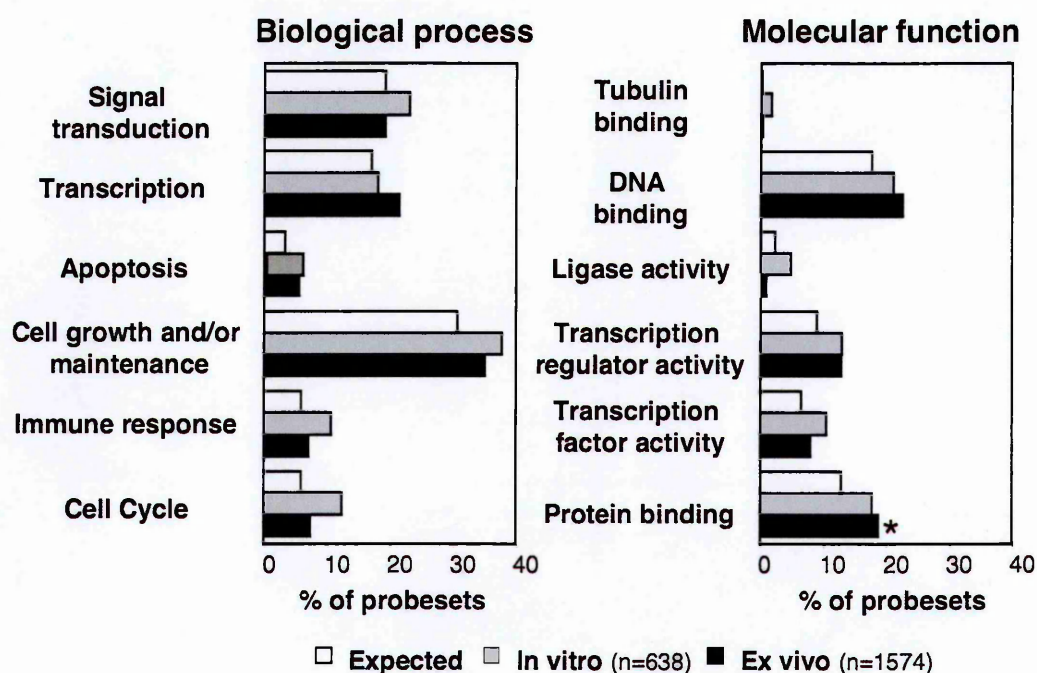
the survival of T cells carrying insertions in the proximity of transcriptionally active regions.



**Figure 18. Correlation between insertion sites and genes expressed in T cells.** Comparison of expression level of all probesets vs probesets of genes hit (inside or <10 kb upstream) by retroviral vector insertion and found in T cells of ADA SCID patients after gene therapy.

### 3.17 No evidence of *in vivo* selection for integrations targeting “dangerous” genes

To understand whether the bias observed in the genomic distribution of RIS was associated to *in vivo* selection of specific integration events, we carried out a functional clustering of genes targeted by retroviral integration based on the Gene Ontology (GO) classification (Ashburner et al., 2000). The expected distribution of genes among the different GO families was compared with the observed distribution of genes hit by integrations (within or  $\pm 30$  kb from genes) in the *in vitro* and *ex vivo* samples. The analysis showed a tendency for a modest, statistically not significant over-representation of several gene categories (signal transduction, cell cycle, cell growth, enzyme activity, immune response) in the pre-transplant CD34<sup>+</sup> cell sample (Fig. 19). The post-transplant dataset displayed a similar profile, with the exception of a slight but significant increase in the frequency of genes encoding protein-binding factors (Fig. 19). Overall, proto-oncogenes were only slightly over-represented (3.1%) compared to their expected frequency in the human genome (2.2%). Collectively, these results indicate that the occurrence of integrations in the vicinity of genes involved in potentially dangerous functions does not necessarily imprint a selective pressure for an *in vivo* expansion to the clones carrying such integrations.



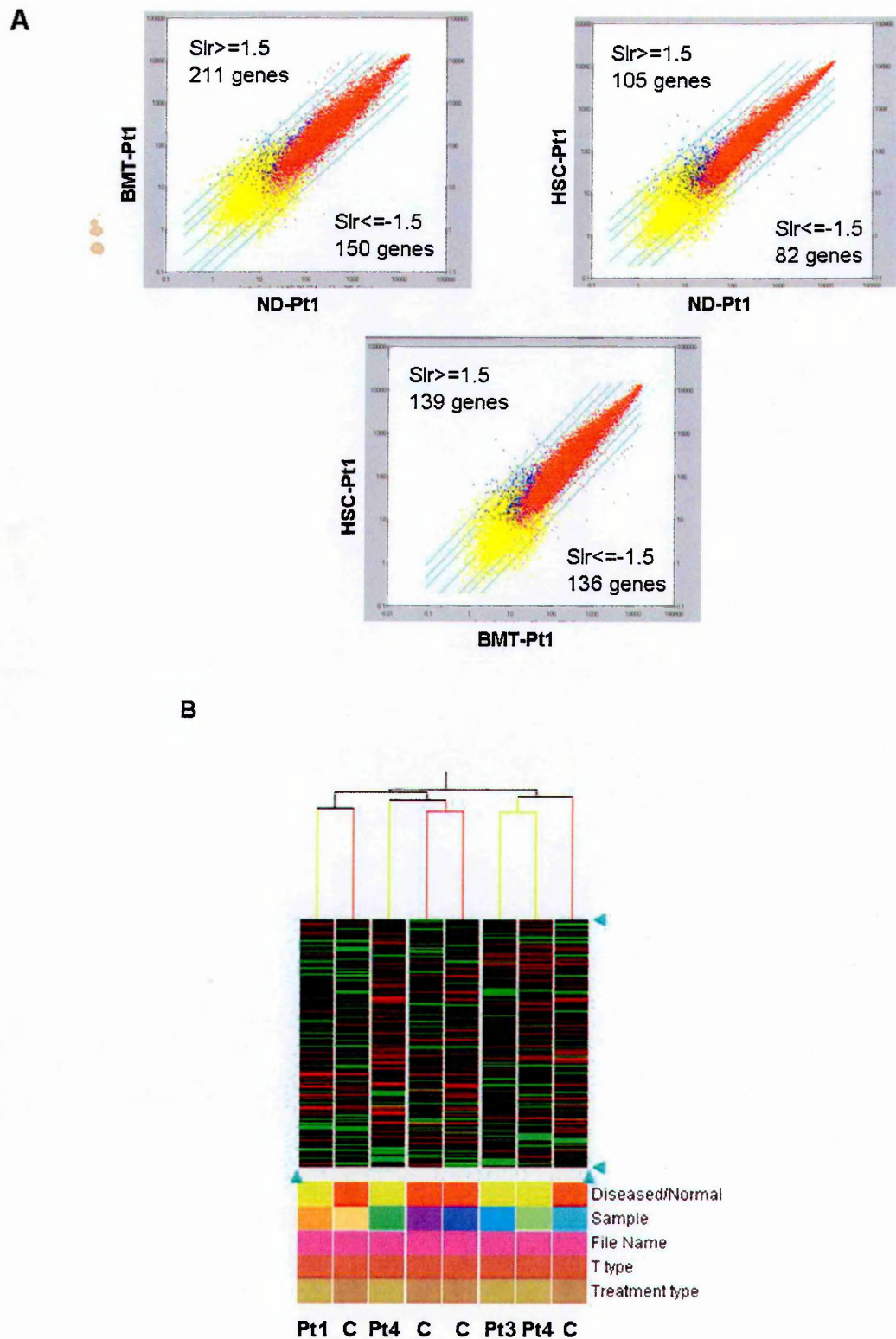
**Figure 19. Gene Ontology (GO) functional clustering of targeted genes.** The probesets of genes targeted (inside and  $\pm$  30 kb upstream or downstream) by retroviral insertion were classified according with Gene Ontology criteria. Each bar refers to the percentage of all of targeted probesets falling within specific gene category. The difference between groups was tested by both Fisher Exact test on Ease score, corrected for multiple comparisons with Bonferroni-Holm correction. The only difference was revealed for “ex vivo” sample in “protein binding” category ( $p_{\text{fisher}}=0.022$ ;  $p_{\text{ease score}}=0.034$ ). Moreover, no major differences were observed in vector integration site distribution and hierarchical clustering between lymphoid and myeloid cells (data not shown).

### 3.18 Normal profile of gene expression in CD4<sup>+</sup> T cells from GT treated patients

We then studied the overall transcriptional profile of purified CD4<sup>+</sup> T cells from three ADA-SCID patients (Pt1, Pt2, Pt3), to assess potential alterations in the transcriptional activity after gene therapy (10-30 months). The expression of >16.000 genes was analyzed by Affymetrix HG-U133A Gene Chip® and compared to those of age-matched healthy controls. For Pt1, we used as control her healthy sibling and another sibling who had received an HLA-identical BMT. As shown in Fig. 20A, there was little difference between the expression profiles of CD4<sup>+</sup> T cells from Pt1 and the family controls: 139 (0.6%) and 105 (0.5%) probe sets showed a significant increase (s.l.r.  $\geq 1.5$ ) in expression compared to the BMT patient and control, respectively, while 136 (0.6%) and 82 (0.4%) showed a comparable decrease (s.l.r.  $\leq 1.5$ ). Similar variations were detected in the comparison of the BMT patient and the healthy control. In an unsupervised comparison (Fig. 20B) of all CD4<sup>+</sup> T-cell samples from controls and patients treated with gene therapy, we did not observed a segregation of samples according to the treatment. Moreover, the hierarchical clustering of samples performed in a supervised analysis showed no significant changes in the expression profile of patients and controls (data not shown). Overall these analyses indicate that there is no difference in the gene expression program of CD4<sup>+</sup> T cells developed after gene therapy with respect to BMT or healthy controls.



**Figure 20**



**Figure 20. Gene expression profile in  $CD4^+$  T cells from patients.** (A) Microarray gene expression analysis of gene therapy (HSC-) Pt1  $CD4^+$  T cells compared to healthy sibling (ND-Pt1) and a BMT-treated sibling (BMT-Pt1) (MAS 5.0 analysis); (B) Unsupervised analysis of  $CD4^+$  T-cell samples from three gene therapy patients (Pts) and four controls (C).

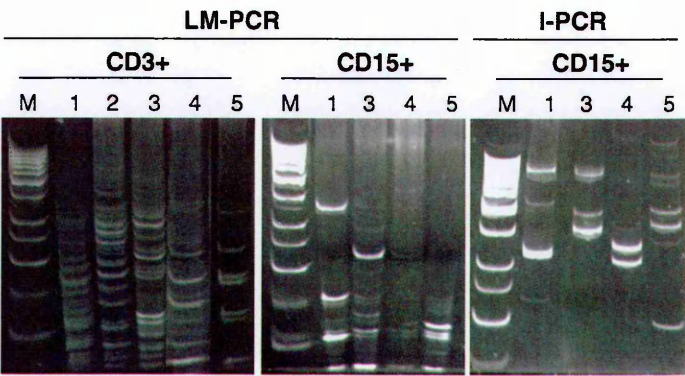
### 3.19 Analysis of vector clonal composition in distinct hematopoietic lineages

To gain a better knowledge of the nature and number of genetically corrected HSC and their differentiated progenies in the ADA-SCID patients, we evaluated integrations in granulocytes, representative of long-term engrafting HSCs, and T cells, which include long-term surviving, mature cells. Among the 496 RIS isolated from *ex vivo* samples from five patients, 124 came from highly purified granulocytes, 399 from T cells (per-patient range: 42-121), and 10 from other cell types. Using RIS as a marker of cell clonality, T-cell populations remained polyclonal throughout the follow-up, in agreement with the polyclonal TCR repertoire observed in the same samples (Fig. 21A and data not shown). In contrast, granulocytes showed a more oligoclonal pattern by gel electrophoresis analysis (Fig. 21A), with fewer but stable integrations retrieved by random cloning in four patients (range 6-84). This may be in part related to the lower proportion of transduced cells engrafted within the myeloid compartment as compared to the lymphoid compartment (Aiuti et al., 2002a). No major differences were observed in vector integration site distribution and hierarchical clustering between lymphoid and myeloid cells, even if the integrations retrieved in granulocytes at early time points after GT (+45 days) behaved more similarly to insertions cloned from pre-infusion CD34+ cells, in terms of preference for distribution inside genes, compared to later time points analysis (> 6 mo; data not shown). While a large numbers of integrations were uniquely detected in single time points, few of them were randomly retrieved at different time points during the follow up (Figure 21B). Remarkably, the analysis performed in various hematopoietic lineages during the follow-up of patients revealed the presence of shared integrants between myeloid (granulocytes) and lymphoid (T and/or B) cells in Pt3 (n=9) and Pt4 (n=4) (Fig. 21B) and confirmed in selected cases by specific PCR. Collectively, these data demonstrate that the engraftment of transduced HSC with multilineage potential has occurred and is maintained in these patients.

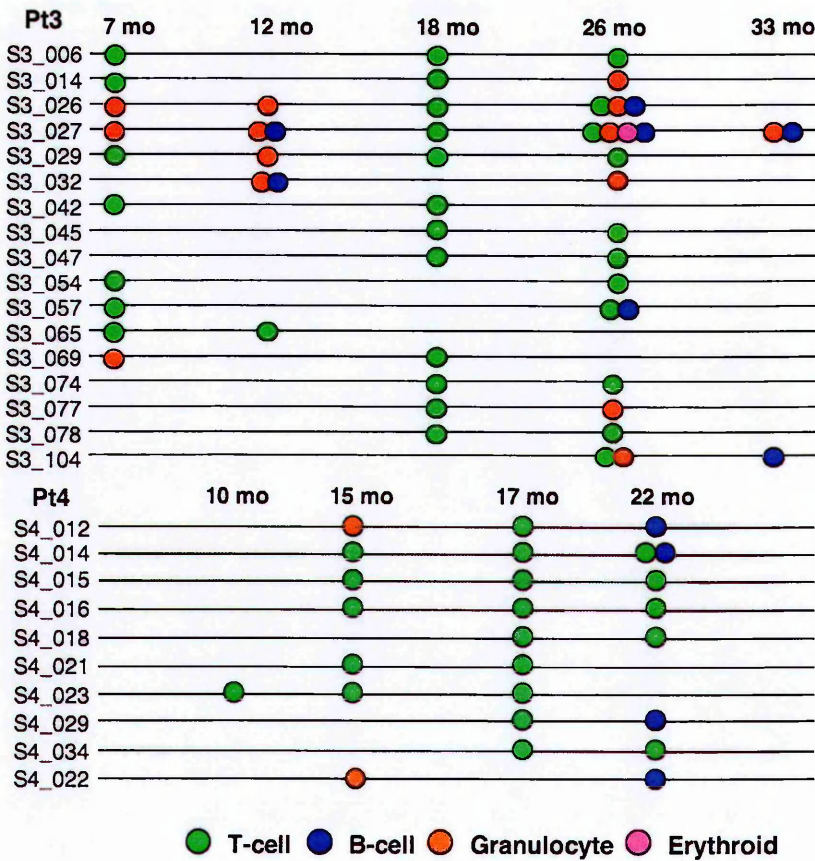


Figure 21

A



B



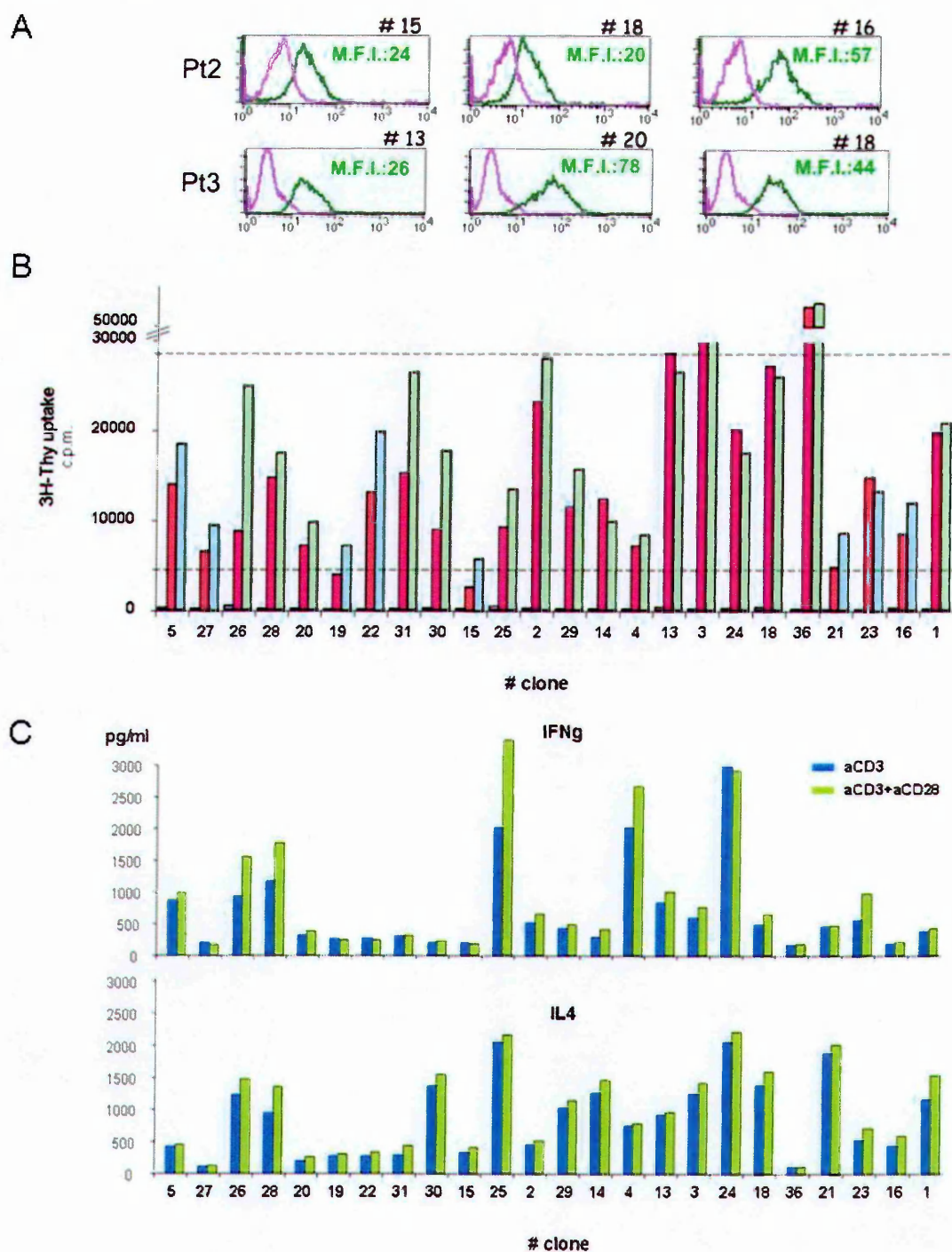
**Figure 21. Vector clonal composition in distinct hematopoietic lineages after gene therapy.** (A) Clonal analysis by LM-PCR and inverse-PCR. Analysis of vector integrations in purified CD3<sup>+</sup> T cells and CD15<sup>+</sup> granulocytes from five ADA SCID patients treated with gene therapy. Nested inverse- or LM-PCR products were analysed by electrophoresis on Spreedex agarose gels with 100bps ladder, as marker (M). In Pt2, who received a low CD34<sup>+</sup> cell dose and displayed poor myeloid engraftment (<0.1%) no RIS could be isolated from granulocytes. The following representative time points are shown: LM-PCR in T cells: Pt1, 20 months (mo); Pt2,

39 mo; Pt3, 26 mo; Pt4, 22 mo; Pt5, 9 mo. LM-PCR for granulocytes: Pt1, 47 mo; Pt3, 33 mo; Pt4, 22 mo; Pt5, 12 mo. Inverse-PCR in granulocytes: Pt1, 5 mo; Pt3, 26 mo; Pt4, 4 mo; Pt5, 3 mo. (B) Evidence for common integrants identified in different cell subpopulation during long-term follow up. Each integration from Pt3 (upper panel) or Pt4 (lower panel) is identified by a unique number. The circles indicate the presence at a given time after gene therapy (M, months) of a specific integration detected by random cloning in the indicated population. Only some integrations found at different time points or in different cell populations are represented. The presence of common integrants was demonstrated also by sequence-specific PCR, which allowed to unequivocally track integrants (i.e.: S3\_027, S3\_026) in different lineages (data not shown), and confirmed the engraftment of transduced HSC with multilineage potential.

### 3.20 Generation of T-cell clones from ADA-SCID patients

To study at the clonal level the effects of vector integration on gene expression and cell function, we have generated T-cell clones by ex vivo limiting dilution from two patients at 18 months after their autologous transplantation with transduced CD34<sup>+</sup> cells (Pt2, 25 clones; Pt3, 28 clones). A complete characterization of these clones was performed in terms of phenotype, TCRVb repertoire, ADA expression/activity and functional properties. All clones were CD4<sup>+</sup> and displayed a high Vbeta family T-cell receptor diversity, as assessed by flow cytometry (data not shown). ADA expression, detected by intracellular staining in all T-cell clones, was within the normal range, but varied in intensity between different clones, suggesting a position dependent expression of the transgene, which we are currently characterizing based on mapped integration sites (Fig. 22A and data not shown). Functional analysis showed normal proliferative responses after TCR/CD28 stimulation and concomitant production of IL-2, IL-4 and IFN- $\gamma$  cytokines for the majority of the clones (Fig. 22B and data not shown).

Figure 22

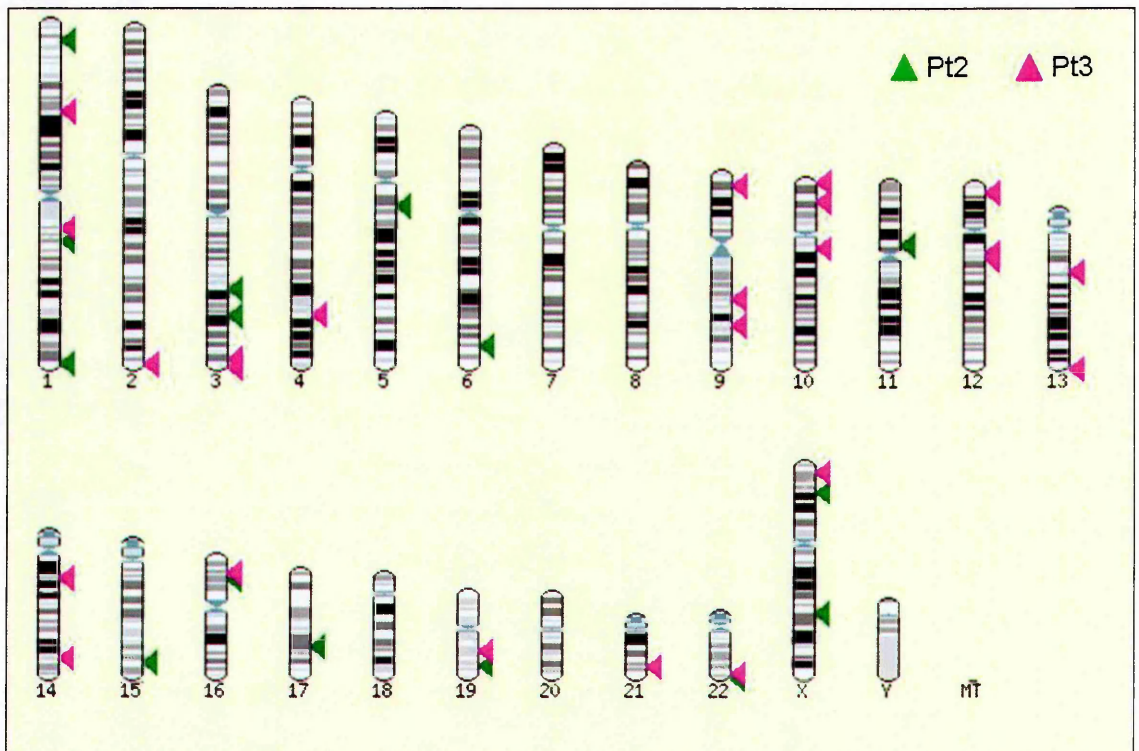


**Figure 22. Characterization of T-cell clones generated from two GT treated ADA-SCID patients.** (A) ADA protein expression was determined by intracytoplasmic staining as described in methods. M.F.I values are indicated. (pink line isotype control; green line ADA). (B) T-cell clones from Pt3 were stimulated with immobilized anti-CD3 (1  $\mu$ g/ml; red bars) in absence or presence of anti-CD28 mAb (10  $\mu$ g/ml; green bars) and  $^3$ H-Thymidine incorporation was assessed after 48h. (C) In parallel, cytokine secretion was evaluated by ELISA in the supernatants collected at 48h.



### 3.21 Analysis of RV integration sites in T-cell clones

Among the clones generated from Pt2, four were negative for the *neomycin* gene marker. In all the other clones, vector-genome junctions were cloned by inverse-PCR, sequenced and mapped onto the human genome (Fig. 23).



**Figure 23. RIS distribution in T-cell clones from two ADA-SCID patients.**

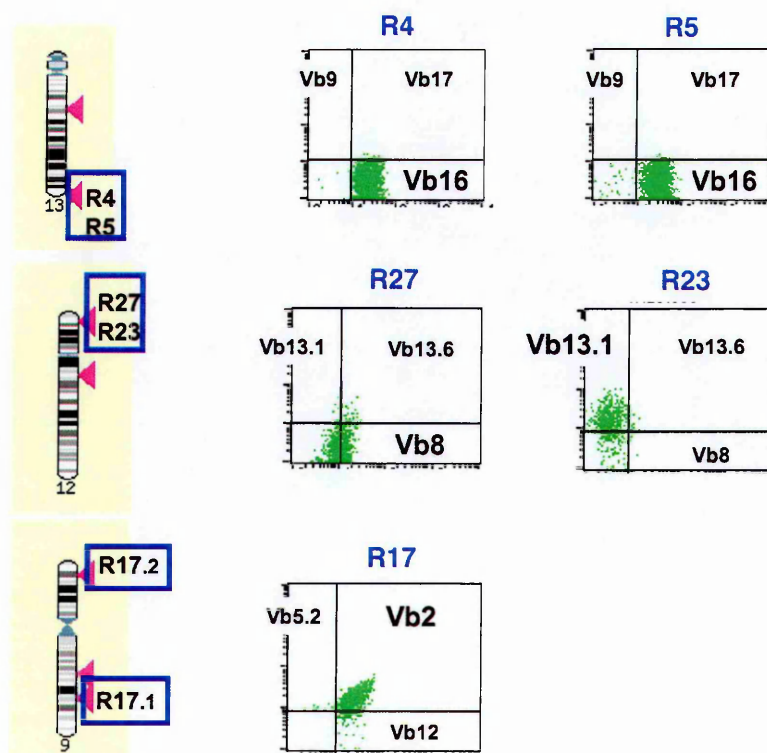
Overall, 39 RIS were unambiguously assigned to a chromosomal position, of which 24 identified from clones of Pt3 and 15 from Pt2. The large majority of the clones contained one vector copy per cell (88%), with few clones carrying two copies (12%), as assessed by quantitative PCR.

As for purified hematopoietic cell populations, RIS distribution analysis showed a cluster of integrations occurring within a 5-kb window upstream or downstream TSSs (36.6%) and a strong preference for gene-dense regions ( $>10$  genes/Mb) (85% RIS).

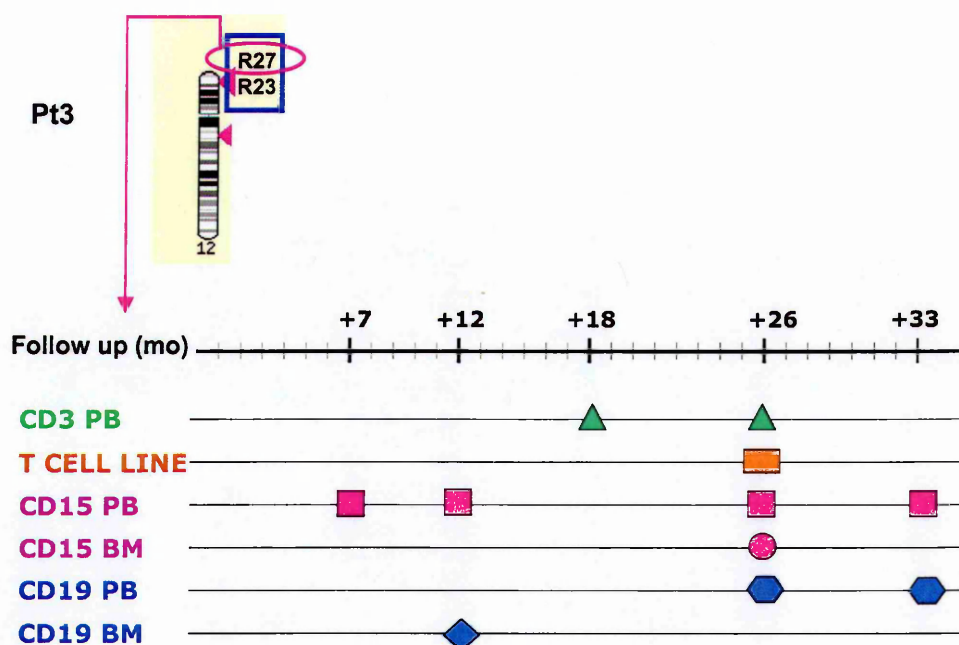
Interestingly, by FACS analysis, we could identify a different Vbeta TCR profile in some clones sharing the same insertion site, thus indicating their progenitor cell origin. In other clones, in depth analysis of Vbeta subfamilies is currently ongoing by CDR3 sequencing. Moreover, we found that some of these clones shared common insertion site with freshly isolated CD3<sup>+</sup> T cells, CD15<sup>+</sup> granulocytes and CD19<sup>+</sup> B cells, confirming that were derived from transduced HSC with multilineage potential.

Figure 24

A



B



**Figure 24. Evidences for stem cell origin of T-cell clones.** (A) FACS analysis of Vbeta profile in T-cell clones from Pt3. Upper panel: two clones sharing the same RIS have same Vbeta profile. Middle panel: two clones sharing the same RIS have distinct Vbeta repertoire. Lower panel: clone having two copies of vector inserted, landed in different genomic regions. (B) One T-cell clone from Pt3 display shared RIS with distinct hematopoietic cell subsets purified *ex vivo* at different time points during the follow up.

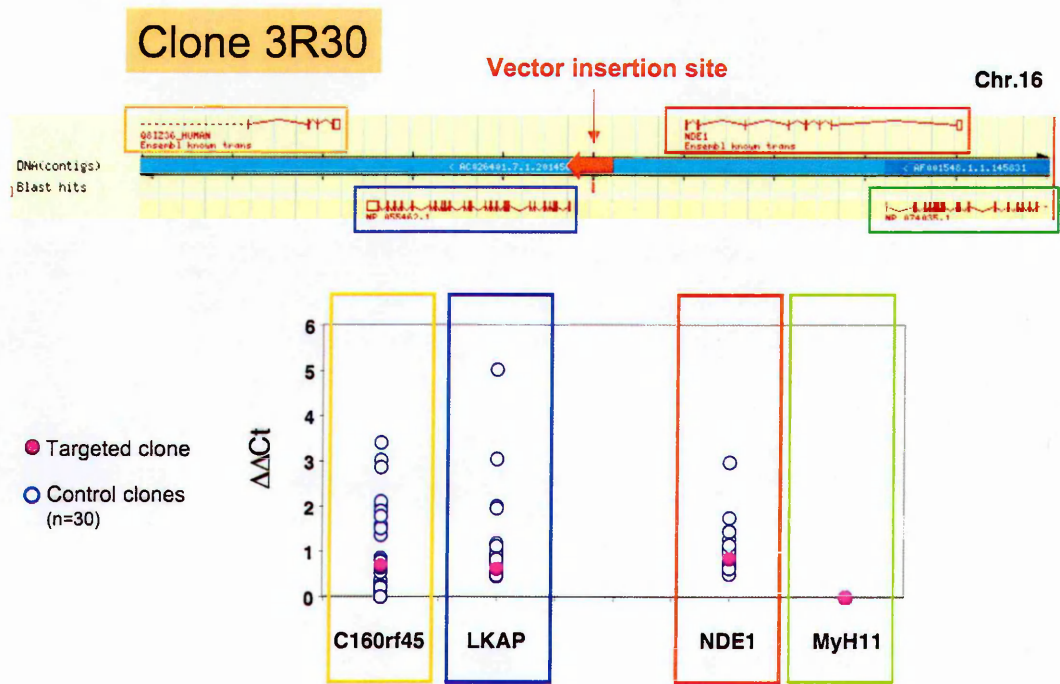
### 3.22 Gene expression analysis of integration-targeted genes

The observed retroviral integration preferences may account for a potential risk of activation and/or deregulation of host cell genes surrounding the integration sites. To directly assess this issue in the context of ADA-SCID GT trial, the expression of 125 genes, mapped within a window of 200 Kb centred on each insertion site, was analyzed by real-time RT-PCR on two microfluidics cards designed to measure gene expression in quadruplicate in the clones carrying the integration(s) and in (a) all other transduced clones (n=49), (b) untransduced clones (n=4), (c) clones from healthy donors (n=4), as controls. Importantly, custom assays corresponding to different transcripts of the same

gene were also included in the analysis, for a total of 145 probes. For each gene, the expression level was measured as relative mRNA quantity following interarray normalization for the level of GAPDH, and plotted as ratio ( $\Delta\Delta Ct$ ) with respect to the average levels, calculated for all but one of the clones i.e. the clone carrying insertion nearby such gene. So far, 105 genes flanking 32 RIS (of which: 9 intragenic and 10 very close ( $<10\text{kb}$ ) to TSS), identified from 30 clones, were analysed. The results of this preliminary analysis showed no alteration in expression level correlated with the nearby vector integration, in agreement with the normal *in vitro* growth and functional behaviour of T-cell clones (Fig. 25 and data not shown).

Figure 25

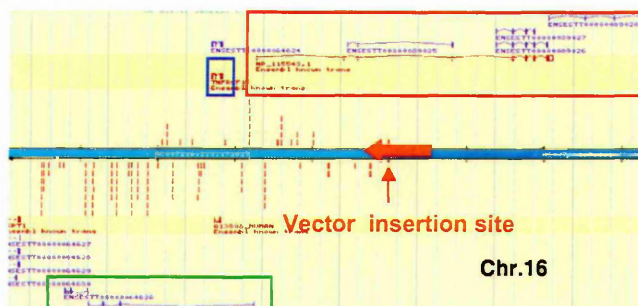
A





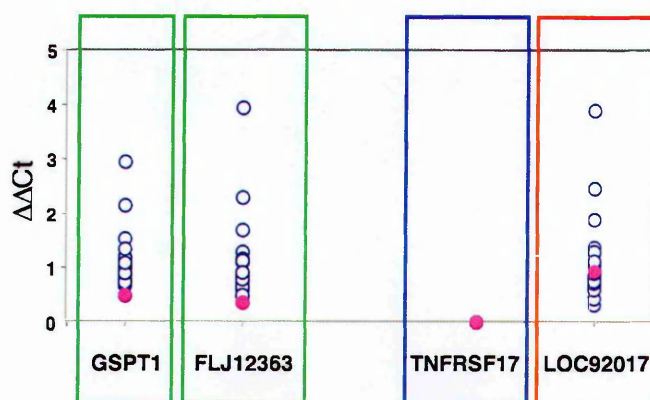
B

Clone 1R16



● Targeted clone

○ Control clones  
(n=30)



**Figure 25. Expression analysis of gene targeted by RIS in T-cell clones.** Quantitative PCR analysis of the expression of genes hit by a RV integration event in individual T-cell clones. (A) Example of an intergenic RIS close to TSS of a gene is shown. RV vector has integrated 5Kb upstream the TSS of LKAP gene, in the same transcriptional orientation, and 20Kb upstream the TSS of NDE1. Expression analysis of other two genes (C16orf45 and MyH11) landed within 100kb window on either side of RIS was also studied. (B) Example of an intragenic RIS. RV vector has integrated in the third intron of LOC92017 gene (distance from TSS 34.4Kb) in the opposite transcriptional orientation. Expression analysis of other three genes (TNFRSF17; FLJ12363 and GSPT1) landed within 100kb window on either side of RIS was also studied. In both cases, after normalization for the level of GAPDH, relative mRNA quantity was plotted as  $\Delta\Delta C_t$  ratio with respect to the average level in all the other analysed clones. The expression value of the clone interested by the hit is indicated by a pink dot, control clones by blue dots. RV orientation is indicated by red arrows.



## **CHAPTER 4 – Discussion**

The results obtained during the course of this PhD project represent new findings, which provide important insights in the pathogenesis of ADA-SCID and at the same time contribute to extend our knowledge on the biology of genetically corrected haematopoietic stem/progenitor cells and lymphocytes, which ultimately impact on the safety and long-term efficacy of gene therapy. Here, the results obtained will be discussed in view of the existing data and in view of their relevance for future clinical and experimental applications.

### **4.1 Characterization of the T cell defect in ADA-SCID patients**

In the present study, we uncover the molecular basis underlying the immunological defect of T cells from ADA-SCID patients. We established T-cell lines from ADA-SCID patients, with a severe clinical phenotype, before their autologous transplantation with gene-corrected CD34<sup>+</sup> cells. For the first time, TCR-mediated activation of ADA-deficient T cells was studied, using a large scale of anti-CD3 mAb doses, combined or not with anti-CD28 mAb. Our study showed that ADA-deficient T cells have a specific defect in response to TCR triggering induced by immobilized anti-CD3 mAb. Importantly, the proliferation defect of ADA-deficient T cells is attributable to both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Although the addition of anti-CD28 mAb to anti-CD3 mAb stimulation enhanced the proliferative response of ADA<sup>-/-</sup> T cells, costimulation was not able to restore normal proliferation in these cells (data not shown). Our study helped to clarify the role of IL-2 deficiency in the inability of ADA<sup>-/-</sup> T cells to proliferate in response to anti-CD3/CD28 mAb. T-cell proliferation is controlled by both TCR/CD28 and IL-2R-mediated signals. Exit from the G1 phase of the cell cycle and entering into the S phase requires IL-2 generated signals (Nourse et al., 1994).

Indeed, triggering of the IL-2 receptor induces key events of G1-to-S phase transition, including up-regulation of cyclin D, down-regulation of p27<sup>Kip</sup>, and hyperphosphorylation of Rb, mediated by PI3K (Brennan et al., 1997). In ADA<sup>-/-</sup> T cells, defective proliferation correlates with reduced IL-2 production. In culture, ADA-deficient T-cell lines require high dose of exogenous IL-2 to grow. Therefore, the inability to secrete sufficient amounts of IL-2 is likely to be the main reason for the poor proliferation of ADA<sup>-/-</sup> T cells. In our study, analysis of the pattern of cytokine production by ADA<sup>-/-</sup> T cells also revealed a strong impairment in the secretion of both Th1 (IFN- $\gamma$  and TNF- $\alpha$ ) and Th2 cytokines (IL-4, IL-5 and IL-10) after TCR/CD28 stimulation. For IFN- $\gamma$  and IL-4 we demonstrated that the low levels in the supernatants correspond to reduced protein synthesis. However, Th1 type cytokines seem to be particularly affected, since more IL-4 than IFN- $\gamma$  was produced upon TCR triggering in ADA<sup>-/-</sup> cells. Similar pattern was consistent with the expression of the corresponding mRNA species, thus indicating a bias occurring at the transcriptional level. The unbalance Th2 *versus* Th1 cytokine production might be related to the higher threshold of activation required for Th1 cytokine gene transcription with respect to Th2 gene, as it has been reported that reduced ERKs activation associates with early IL-4 production and Th2 differentiation (Jorritsma et al., 2003). However, defective IFN- $\gamma$  production was also present upon strong stimulation with TPA plus ionomycin, suggesting that it might likely derive from an intrinsic developmental defect. Analysis of T-bet and GATA-3 mRNA levels in resting and activated CD4<sup>+</sup> T-cell lines revealed a significantly reduced expression of T-bet in ADA<sup>-/-</sup> compared to control cells. Therefore, it is likely that the reduced T-bet expression contributes to the defective IFN- $\gamma$  gene transcription in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells, as T-bet is one of the key factors involved in IFN- $\gamma$  production by CD4<sup>+</sup> T cells (Szabo et al., 2002) (Szabo et al., 2000) and in Th1 differentiation. T-bet was shown to positively regulate its own expression by a

mechanism involving IFN- $\gamma$ -induced STAT1 activity (Afkarian et al., 2002). Therefore, defective production of T-bet and IFN- $\gamma$  in ADA<sup>-/-</sup> CD4<sup>+</sup> T cells could also result from the lack of a positive feedback. On the other hand, ADA<sup>-/-</sup> CD4<sup>+</sup> T cells were able to express normal levels of the transcription factor GATA-3 mRNA, which positively regulate IL-4 gene transcription and Th2 commitment (Ouyang et al., 2000). In this view, our results are in agreement with the clinical phenotype of ADA-SCID patients, characterized by recurrent allergic airway inflammations and markedly elevated IgE (Hirschhorn, 1999). Moreover, our *in vitro* results are relevant for the understanding of the cellular mechanisms associated ADA-SCID immunodeficiency. Reduced IL-2 and IFN- $\gamma$  production by activated CD4<sup>+</sup> T cells, and overall impairment in Th1 differentiation is likely to result, *in vivo*, into poor help to CD8<sup>+</sup> T cells during the establishment of immune response against viral pathogens. The precise requirements of CD4<sup>+</sup> T cells for the generation and survival of memory CD8<sup>+</sup> T cells has been deeply investigated (Rocha and Tanchot, 2004). While the activation and acquisition of effector functions by CD8<sup>+</sup> T cells during primary immune response do not require CD4<sup>+</sup> help at the moment of priming, the capacity to undergo clonal expansion upon Ag re-exposure is programmed via interaction with CD4<sup>+</sup> T cells and APC during primary stimulation (Janssen et al., 2003). It has been proposed that help by CD4<sup>+</sup> cells confers competitive “fitness” to memory CD8<sup>+</sup> T cells, thus contributing to the generation of long-term surviving memory CD8<sup>+</sup> T cells (Johansen et al., 2004). We can speculate that, in ADA-SCID patients, the inability to efficiently clear viral infections results from both an intrinsic CD8<sup>+</sup> T cell defect and from inadequate help by Th1 cells.

The above results on cytokine genes transcription strongly suggest that severely compromised effector functions in ADA-deficient T cells resulted from a defective ability to transduce activating signals from the membrane TCR/CD28 to the cell nucleus. We analyzed one of the main signaling events triggered by TCR/CD28

engagement in ADA-deficient CD4<sup>+</sup> T cells. Our observation that phosphorylation of MAPK ERK1/2 is generally defective in ADA<sup>-/-</sup> cells, compared to healthy donors, is in accordance with previous data obtained in freshly isolated thymocytes from ADA knock-out mice, in which a reduced phosphorylation of the upstream molecule CD3  $\zeta$  chain has been reported (Apasov et al., 2001).

In agreement to the fact that proximal TCR signaling is impaired in ADA<sup>-/-</sup> T cells, a reduction in the activation of CREB was observed early after TCR/CD28 triggering. This event is linked to AP-1 proteins expression and IL-2 production. Indeed, CREB is involved in the induction of c-jun, c-fos, Fra-2, and FosB after TCR cross-linking (Kuo and Leiden, 1999). Therefore, our results are consistent with a model in which the absence of functional CREB, in ADA<sup>-/-</sup> T cells, causes defective induction of AP-1 and IL-2 and subsequent proliferation impairment. In addition, the production of IL-2 is possibly determined by the ratio between pCREB and CREM, a family-related transcriptional repressor, which occupies the -180 site of the *IL2* promoter (Tenbrock et al., 2003). Since the reduced CREB phosphorylation may limit its nuclear translocation and binding to the *IL2* promoter, it is possible that an increase CREM ligation to the promoter-binding site might be involved in the block of IL-2 production observed in ADA<sup>-/-</sup> cells. It would be important to study the accessibility of IL-2 promoter in ADA-deficient cells or the *in vivo* binding of these transcription factors.

Analysis of I $\kappa$ B $\alpha$  phosphorylation in ADA-deficient cells, after TCR/CD28 triggering, showed that at the time points evaluated the levels of phosphorylation were undetectable or markedly reduced compared to controls. I $\kappa$ B $\alpha$  is normally degraded after phosphorylation by the proteosome pathway (Karin and Ben-Neriah, 2000). At the later time point evaluated (2h) reduced levels of I $\kappa$ B $\alpha$  phosphorylation could reflect reduced targeting to degradation and hence low level of NF $\kappa$ B translocated in the

nucleus. It would be therefore interesting to further investigate NF $\kappa$ B activity and localization of NF $\kappa$ B subunits in the nucleus of ADA<sup>-/-</sup> cells.

Collectively, these results suggest that rather than controlling a single pathway downstream the TCR, the immune defect in ADA-SCID may involve multiple pathways converging towards a defective induction of the transcriptional machinery.

Interestingly, reduced methylation of Vav-1, a regulator of calcium increase, as well as impaired ERKs activation and nuclear transcriptional events linked to NFAT and NF $\kappa$ B have been recently associated to drug-induced inhibition of SAHH enzyme (Blanchet et al., 2005). Block in SAHH activity, classically considered an hallmark of ADA-SCID (Hershfield, 2001), has been observed in ADA<sup>-/-</sup> T cells. These findings strongly support the notion that an intracellular toxicity persists in ADA-deficient T cells, which developed *in vivo* in a poisoned environment, even under culture conditions. However, in order to directly investigate the role of altered purine metabolism in the immune dysfunction, we exposed T cells to ADA-SCID *in vivo* -like condition.

#### **4.2 Role of altered purine metabolism in the defect of ADA-deficient T cells**

Although available evidence suggests that the metabolic basis for ADA-deficient immunodeficiency is related to the physiological impact of the ADA substrates, there remains uncertainty about the relative contribution of Ado- *versus* dAdo-related mechanisms to immune dysfunction. Historically, Ado-induced cAMP formation in lymphocytes was one of the first hypothesis offered to explain the lymphopenia observed in patients affected by ADA-SCID. However, elevated lymphocyte cAMP was not demonstrated *in vivo*, and *in vitro* Ado lymphotoxicity could be explained by its uptake and intracellular metabolism (Hershfield, 2001).

Despite the limited knowledge regarding its own physiological function, dAdo-related mechanisms have been generally proposed as the primary cause of lymphotoxicity in ADA-SCID. Indeed, it is well established the correlation existing between the severity of the disease and the concentrations of dAdo and its nucleotides derived (dAXP) in erythrocytes and excreted fluids. Moreover, while dAdo accumulation is present from birth, the finding of high plasma and urinary Ado in late presenters or patients underwent BMT, support the concept that dAdo, but not Ado, is toxic to the immune system (Hershfield, 2001) (Hirschhorn, 1999). Therefore, understanding the molecular mechanisms, linking elevated dAdo to immune dysfunction, acquires growing importance as it may also provide a rationale prediction of the relationship between clinical manifestations and molecular defects. With respect to Ado, this metabolite displays a lower affinity for adenosine receptor (Fredholm et al., 2001). Whether the local overload of dAdo may overcome this reduced activity enabling the initiation of the transmembrane signaling has not been explored. Our study describes for the first time a dAdo-related mechanism responsible for T cell dysfunction in ADA-SCID, which is independent from dAdo intracellular poisoning but consistent with the engagement of an aberrant extracellular receptor signaling. We have demonstrated that exogenous dAdo, at the concentrations resembling the *in vivo* accumulation (Aiuti et al., 2002a) (Aiuti A. manuscript in preparation), act on the A<sub>2A</sub> receptor and suppress activation and effector functions of ADA-deficient T cells. Our data are consistent with a key role of cAMP/PKA in the dAdo-mediated inhibitory effect. The role of cAMP/PKA pathway in the physiological control of inflammatory T cell responses has been extensively characterized (see chapter 1). However, in the context of defective dAdo-metabolizing enzyme, where the intracellular cAMP levels would be reasonably increased, this regulation may be overstated, leading to the immune defect. Indeed, interference with the proximal biochemical events following T-cell receptor-triggering and inhibition of

downstream effector functions has been demonstrated by pharmacologic increase of intracellular cAMP with the use of A<sub>2A</sub> receptor agonist (Huang et al., 1997; Skalhogg et al., 1992) (Butler et al., 2003) (Lappas et al., 2005). Phosphorylation of ERK1/2 MAPK was notably abrogated in ADA-deficient T cells stimulated in presence of dAdo, suggesting that an ADA-deficient environment *in vivo* is not likely conducive to normal TCR signaling. Moreover, it predicts that other events of TCR signaling may be inhibited by dAdo in conditions of ADA-deficiency. In T cells, ERK1/2 MAPK is involved in the induction of AP-1 activity by promoting Fos expression (Hunter and Karin, 1992). ERK1/2 is linked to IL-2 and IFN- $\gamma$  production (Badou et al., 2001) (Koike et al., 2003) and regulates cell proliferation through direct stimulation of DNA synthesis (Graves et al., 2000). These activities require translocation of phosphorylated ERK1/2 to the nucleus. Therefore, we can reason that, in ADA-SCID patients, inhibition of ERK1/2 activation is responsible for the suppressed effector functions.

Preventing MAPK ERK1/2 activation, dAdo may drive TCR-stimulated T cells towards an anergic rather than an immunogenic response. Recent studies have challenged the biochemical definition of the anergic state, showing that anergic T cells are incapable of activating lck, ZAP-70, phosphorylating TCR  $\zeta$  chain, activating Ras and ERK and transactivating AP-1 and NF-AT (Cho et al., 1993) (Madrenas et al., 1995) (Boussiotis et al., 1996) (Fields et al., 1996) (Mondino et al., 1996) (Kang et al., 1992). The cAMP/PKA pathway hyperactivation and SAHH inhibition may play a causative role in the induction and propagation of this anergic state, as they can affect mostly of the above mentioned biochemical events, generally delivering off signals (Torgersen et al., 2002) (Blanchet et al., 2005). Therefore, such a mechanisms might be responsible for preventing T-cell activation and expansion during Ag-driven immune responses and may offer a better comprehension for the clinical autoimmune

manifestations observed in ADA-SCID patients (Kohn DB, 1996) (Ozsahin et al., 1997).

Previous results in ADA knockout mouse model have indicated Ado but not dAdo as involved in the inhibition of TCR-triggered events leading to T cell survival (Apasov et al., 2001), thus suggesting an exclusive dAdo-related intracellular mechanism of action. It is conceivable that these opposing results are due to the different cell-type, which may exhibit different metabolic pathways and sensitivity. Indeed, in thymocytes the pathway of nucleoside phosphorylation is known to be particularly active, thus playing a major role in the dAdo-mediated toxicity. However, our observation that the inhibitor of nucleoside transport system enhanced the dAdo-mediated inhibitory effect on TCR-dependent proliferation of ADA<sup>-/-</sup> T cells argues in favour of two simultaneously cooperating mechanisms in the lymphotoxicity induced by dAdo in ADA-deficient T cells: the extracellular signaling through adenosine receptors and the intracellular metabolism.

The cause of T lymphopenia, in ADA-SCID, has been primarily attributed to induction of apoptosis by elevated levels of ADA metabolites (Kizaki et al., 1990) (Benveniste and Cohen, 1995). Indeed, a relatively high fraction of T lymphocytes isolated from the peripheral blood of ADA-SCID patients treated with PEG-ADA are committed to apoptosis (Malacarne et al., 2005), suggesting that this is a relevant mechanism of lymphopenia even in patients undergoing enzyme replacement therapy. In our study, we found that ADA-deficient T cells exhibit an increase susceptibility to apoptosis when exposed to high concentrations of both ADA substrates. Thus, these results indicate that a general deregulation of T cell function/homeostasis and a direct T cell depletion could be responsible for the complex picture of ADA-SCID clinical manifestation, such as immunodeficiency and autoimmunity.



### 4.3 Efficacy of HSC gene therapy

The results presented in this PhD thesis complement and extend the clinical evidences indicating the efficacy of HSC-based gene therapy in correcting both the metabolic and immune defect of ADA-SCID patients.

In untransformed T-cell lines generated from patients after gene therapy ADA expression was at therapeutic level, enabling the full correction of the secondary additional metabolic defect. Normalization of SAHH activity was pointed out as the most sensitive indicator of normalization of metabolite concentrations and, more recently, as prerequisite for full T-cell activation and immune responses (Blanchet et al., 2005). Accordingly, gene-corrected cells displayed restored proliferative responses and the normal ability to produce cytokine upon TCR triggering, with no particular skewing toward Th1- or Th2-like phenotype. The significant but not exclusive IFN- $\gamma$  production in T cells of all patients implies that these lymphocytes are not in anergic state and the transduction of intracellular activation signals is not defective. Since IFN- $\gamma$  is released by “effector-memory” T cells, it is likely that cells producing this cytokine *in vitro* had responded properly to *in vivo* antigenic stimulation by becoming terminally differentiated effectors.

The biochemical events following TCR triggering occurred properly leading to correction of the downstream pre-transcriptional events. Collectively, these results have an important impact on the safety of gene therapy approach. T-cell lines generated *ex vivo* after GT maintained a polyclonal phenotype and a normal, cytokine-dependent, *in vitro* growth and functional behavior, indicating the absence of any gene transfer-related toxicity.

While in ADA-deficient cells exposure to dAdo induced a strong inhibition of T cell activation, gene-corrected cells were only minimally affected as healthy control

cells. Moreover, gene-corrected T cells displayed a normal sensitivity to apoptosis induced by extracellular and intracellular mechanisms, further confirming the efficacy of ADA expression mediated by retroviral gene transfer in restoring normal metabolic functions.

Our findings are consistent with a key role of the extracellular isoform of ADA in hampering the toxic effects exerted by dAdo in gene-corrected cells. Firstly, the A<sub>2A</sub> selective receptor agonist but not dAdo induced comparable level of intracellular cAMP in GT and ADA-deficient cells, leading to inhibition of proliferative responses. Secondly, the poorly hydrolizable adenosine analogues, 2-chloro-deoxy-adenosine (2-CdA) and 2-chloro-adenosine (2-CA) (data not shown), but not dAdo similarly triggered apoptosis in GT and ADA-deficient T cells. However, providing a formal demonstration that gene therapy allows the correct translocation of the soluble form of functional ADA to the cell membrane would be extremely important.

#### 4.4 Clonal analysis and *in vivo* dynamics of hematopoietic stem cells and lymphocytes in ADA-SCID patients

In addition to their therapeutic function, stably integrated proviruses represent a unique biomarker to study the biology, dynamics and clonality of HSC and their progeny after transplantation. Indeed, while T cell population includes also long-term surviving mature cells, due to the different half-life, granulocytes are representative of long-term engrafting hematopoietic stem/progenitor cells. Clonal analysis in the T-cell compartment demonstrated a high number of distinct insertion sites, in agreement with the complexity of the T-cell repertoire following immune reconstitution in all patients. This heterogeneity was confirmed by the analysis on 49 T-cell clones generated *ex vivo* from Pt2 and Pt3 18 months post-GT, 39 of which had distinct retroviral integrations. T-cell clones displayed a high TCR diversity, and carried either one (87%) or two (13%) vector copies per cell. Less integrations were retrieved in the myeloid compartment as compared to T lymphocytes. These results reflect the 1-2 log difference in HSC vs lymphoid engraftment observed in these patients (Aiuti et al., 2002a) (Aiuti et al., manuscript in preparation) and the survival advantage of ADA-expressing lymphocytes. Importantly, the presence of shared integrants in myeloid and lymphoid cells confirms that our gene transfer protocol, combined to low-dose chemotherapy, is adequate to the objective of achieving engraftment of genetically corrected, multipotent HSC. Contamination of T-cell derived integrations in myeloid cells are unlikely, since cells were highly purified by cell sorting and no integrations could be retrieved in multiple myeloid cell samples which carried <0.1% transduced cells (Pt2), despite the large number of RIS present in peripheral blood lymphocytes. These results differ from those obtained in an ADA-SCID GT trial based on retrovirally transduced umbilical cord blood CD34<sup>+</sup> cells (Schmidt et al., 2003). In this study, one patient showed poor engraftment of transduced HSC and as few as one progenitor cell clone contributing to

long-term T lymphopoiesis (Schmidt et al., 2003). This difference could be explained by the fact previous studies did not use pre-transplant conditioning and maintained patients on enzyme replacement therapy with PEG-ADA, likely abrogating the selective advantage for gene-corrected cells. On the other hand, the existence of transduced progenitor cell clones with myeloid and lymphoid potential has been recently obtained in the SCID-X1 gene therapy trial (Schmidt et al., 2005), indicating that pre-conditioning is not an absolute requirement for HSC engraftment. In this context, further longitudinal analyses of specific vector integrants in multiple lineages would be important to provide a direct estimate of the number of transduced progenitors that contribute to long-term lymphopoiesis or myelopoiesis.

#### **4.5 Vector integration sites analysis in ADA-SCID GT trial**

Our study provides a comprehensive analysis of the profile of retroviral vector integrations in hematopoietic cells before and after transplantation into ADA-SCID patients. We found that both *in vitro* and *ex vivo* integrations showed a non-random distribution of RIS, with a tendency to hit transcriptional units and a strong preference for gene-dense regions. In accordance with previous studies *in vitro* and in animal models (Mitchell et al., 2004) (Hematti et al., 2004), targeted genes, and particularly those hit near start sites, tended to be expressed, or highly expressed, in CD34<sup>+</sup> cells at the time of transduction. These data reinforce the concept that also in stem and progenitor cells the viral integrating machinery interacts preferentially with factors bound in or near genes, as previously observed in transduced haematopoietic cells from non-human primates (Hematti et al., 2004). However, our results show that, following *in vivo* repopulation, there is a tendency to select against integrations occurring inside transcriptional units, and for those occurring in the vicinity (<30 kb) of transcription start sites. This may reflect a growth advantage for clones expressing adequate ADA

levels, in which integration near promoters could increase the chance of a productive interaction between the vector and the cell transcriptional machinery. Indeed, *ex vivo* integrations retrieved from T cells were enriched for highly expressed genes, suggesting the occurrence of an *in vivo* selective pressure for vector-ADA expression. Alternatively, retroviral insertions might have influenced the expression of genes involved in cell expansion or clonal dominance. This explanation appears unlikely, since, in contrast to previous observation in animal models (Montini et al., 2006) (Kustikova et al., 2003), we observed a comparable frequencies of CIS retrieved in pre- and post transplant samples and no *in vivo* skewing towards integrations in genes controlling crucial steps in self-renewal or survival of hematopoietic cells, such as cell cycle, proliferation, or signal transduction. Moreover, there were no major differences observed in vector integration site distribution and hierarchical clustering between lymphoid and myeloid cells (data not shown). Similar findings were observed genetically modified T cells in from leukemic patients treated with retrovirally-transduced donor lymphocytes, with no evidence of clonal selection after transplantation *in vivo* (Recchia et al., 2006). Thus, the 3-4 fold potential increase in the genotoxic risk predicted by the non-random genomic distribution of retroviral integrations appears to have a limited impact in the ADA-SCID case, confirming that the actual risk of insertional oncogenesis is several order of magnitude lower than the simple risk of hitting a potential oncogene ( $10^{-3}$  to  $10^{-2}$ ) (Baum et al., 2006). In agreement with other MLV insertion site analysis, we observed the occurrence of regional clustering and recurrent integration sites.

Chromosomal clusters of insertions were located not only in gene high density regions, but also in relatively low density ones (12p13.32, 17q23.2, 6q25.3, 11p11.2, 15q26.1), implying the existence of peculiar substructures within chromosomal regions that favor retroviral integration. Comparison of RIS data from different clinical trials

should allow identifying genomic regions that are preferential targets of retroviral vectors, independently from the cell target and the therapeutic gene.

#### 4.6 Comparison with other GT clinical trials

A comparison of our RIS collection with that of a recently reported CGD gene therapy trial (Ott et al., 2006a) reveals that 9.7% of the RIS detected *in vitro* (n=24) or *in vivo* (n=46) are in/near the same genes. However, unlike the CGD trial, in which cell clones containing activating insertions in *MDS1-EVII* gene became predominant over time, we detected only a single non-persisting integration inside *MDS1-EVII*. RIS within this genomic locus have also been recently found to be overrepresented (1.9% of integrations), albeit not clonally expanded, in a retroviral-based preclinical study in primates (Calmels et al., 2005). Of notice, 2 other genes found once in our database (*ATXN1*, *RUNX1*) were considered as frequently hit genes in the primate study (Hematti et al., 2004).

The lack of over-representation and/or expansion of *MDS1-EVII* integrations in our study may have several causes, such as i) the use of BM-derived CD34<sup>+</sup> cells, which have different properties (expression of retroviral receptors, phenotype, cell cycle status) compared to the mobilized PB cells used for CGD patients and primates, and/or ii) the use of an LTR with substantially less enhancer activity in stem and myeloid cells compared to that of the spleen focus-forming virus (SFFV; (Ott et al., 2006a)) used in the CGD trial. Interestingly, 34 RIS (4.8%) from our samples are in common with a collection of 322 RIS retrieved from PB T lymphocytes transduced with an MLV-derived vector *in vitro* and *ex vivo* (Recchia et al., 2006). Overall, these results reveal the existence of a common fingerprint of vector insertions in human cells, which should be further defined in larger collections of RIS and in multiple clinical trials.

A most striking finding of our study is the over-representation of *LMO2* insertions. *LMO2*-related integrations were detected in pre-transplant CD34<sup>+</sup> cells (1/212), as well as in granulocytes (2/124) and T cells (3/399) after transplantation.

Remarkably, integrations in the proximity of *LMO2* were found also in cord blood-derived CD34<sup>+</sup> cells (2/450, not shown) as well as in PB cells (2/765) from CGD patients (Ott et al., 2006). Taken together, these data indicate that *LMO2* is a “hot spot” for retroviral integration in human CD34<sup>+</sup> cells. Since *LMO2* is highly expressed in CD34<sup>+</sup> cells and lies in a gene-dense region, this bias may just reflect the general preferences of gammaretroviral vector. However, specific properties of the *LMO2* locus, such as binding of transcription factors or other chromatin characteristics, may further increase its targeting by the retroviral vector integration machinery. Indeed, the *LMO2* locus (11p13) is a fragile chromosomal site (FRA11E) (Bester et al., 2006), and is a major site of aberrant trans-V(D)J recombination between immune loci in thymocytes (Marculescu et al., 2002), a typical marker of genomic instability.

The lack of *in vivo* expansion of clones carrying *LMO2* integrations and the absence of *LMO2* overexpression, at least at the level of bulk cell population, indicate again that insertions landing into potentially dangerous genomic sites are not sufficient *per se* to induce proliferative advantage in T cells *in vivo*, confirming that multiple cooperating events, deregulating various cellular pathways, are required to promote oncogenic transformation in humans (McCormack and Rabbitts, 2004). It is tempting to speculate that the difference between the ADA-SCID and the SCID-X1 studies may be related to the SCID-X1 genetic background (Shou et al., 2006) or the role of the therapeutic transgene. Indeed, where ADA is a housekeeping enzyme of the purine metabolic pathway and its expression has not been associated with tumor formation, therefore providing an excellent “neutral” system for fully assessing the role of

insertional mutagenesis in HSC,  $\gamma c$  is a potentially oncogenic growth factor receptor (Dave et al., 2004) (Woods et al., 2006).

This is in agreement with the fact that, with the exception of the SCID-X1 study, no other adverse event related to gene transfer have been in GT clinical trials or in long-term observation studies in large animal models (Kohn et al., 2003) (Kiem et al., 2004). On the other hand, we cannot provide conclusive evidence that the integrations detected in our ADA-SCID patients influenced *LMO2* expression at single cell level. The probability for a retroviral vector insertion to induce overexpression of a proximal gene was estimated to be 20% in the context of T cells (Recchia et al., 2006). However, we were unable to isolate T-cell clones containing *LMO2* integrations to directly test this hypothesis in our patients.

#### **4.7 Ongoing studies evaluating the potential risk of transcriptional perturbation**

A crucial parameter in the calculation of the potential risks of gene transfer is the frequency by which an integrated provirus leads to activation, or deregulation, of gene expression. T-cell clones generated *ex vivo* from single-cell represent a unique tool to directly establish a relationship between integration sites and gene expression pattern of lymphocytes. We have generated 49 T-cell clones from two patients, identified insertion sites and evaluated the expression of nearby genes. In a preliminary analysis of 105 genes flanking 29 RIS, either intragenic or intergenic close to TSS of a gene, we found no alteration in expression level correlated with the nearby vector integration, in agreement with the normal, cytokine-dependent, *in vitro* growth and functional behavior of T-cell clones. Although incomplete, our results seem to be slightly different to those obtained by Recchia et al., which estimated the frequency of transcriptional perturbation to be 20% (Recchia et al., 2006), and indicate that insertional gene deregulation, if any, likely manifest in a competitive disadvantage, leading to extinction of the affected cell.



Accordingly, no evidence of clonal dominance or preferential survival was observed in relatively long follow-up of ADA-SCID patients. The transcriptional stability of gene-corrected cells was assessed also in polyclonal T cell population. Expression analysis of >16,000 genes by Affymetrix GeneChip® showed no significant difference with respect to healthy controls, suggesting that our gene therapy protocol does not cause major alteration in the gene expression program of T lymphocytes developed after transplant. Completion of these studies will provide critical information on the potential transcriptional interference occurring at the vector integration site in a relevant cell source from a successful gene therapy trial for ADA-SCID.

In summary, our data show that transplantation of ADA-transduced HSC does not result in *in vivo* skewing or selection of expanding or malignant clones, despite the occurrence of insertions near potentially oncogenic genomic sites. In addition, transduced progeny display a normal gene expression profile, phenotype and immune functions. These results, combined to the relatively long-term follow-up of patients, indicate that retroviral-mediated gene transfer for ADA-SCID has a favorable safety profile while the safety of other trials may be influenced by the nature and regulation of the therapeutic gene as well as by disease- and/or protocol-specific factors. Thus, for selected genetic diseases requiring transfer of genes potentially involved in the oncogenic cascade, further development of potentially safer tools, such as self-inactivating lentiviral or retroviral vectors combined to the use of physiologically controlled promoters, will be required to reduce the potential risk associated with gammaretroviral vectors.

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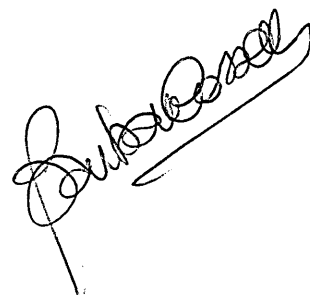
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A handwritten signature in black ink, appearing to read 'D. Zufferey', is written diagonally across the bottom right of the page.